OBITUARY NOTICES

GEORGE BARGER

1878–1939

THE sudden death on 6 January 1939 at the age of 60 years of Prof. George Barger removes one of the most distinguished of the group of chemists and physiologists who were responsible for the formation of our Society and to whose work we owe in large measure the development of Biochemistry in this country.

George Barger was born in Manchester in 1878, his father Gerrit Barger being a Dutch engineer and his mother English. He received his school education in Utrecht where the family settled in his early childhood. At the age of 16 years he chose to return to England to complete his education and entered University College, London, with a scholarship which he won on the University Matriculation Examination; here he commenced his studies for the London B.Sc., later proceeding with a scholarship to King's College, Cambridge. As an undergraduate student Barger was almost equally interested in chemistry and botany, and he was placed in the first class in both of these subjects in Part II of the Natural Sciences Tripos on the last occasion on which more than one subject could be offered for this examination.

After leaving Cambridge Barger was appointed to a demonstratorship under the late Prof. Errera in the department of Botany in the University of Brussels. During the two years which he spent in Brussels he worked at two problems, the chemistry of the glucoside saponarin, and the micro-method of molecular weight determination which bears his name; the latter represents the first of his scientific contributions to find application in the field of biological chemistry and it is significant in indicating his early appreciation of the importance of micro-methods in the development of this branch of science.

In 1903 Barger returned to England and entered the Wellcome Physiological Laboratories as chemist. The decision to take this post, although it was only reached after considerable hesitation, proved to be of the greatest importance for his subsequent career; it brought him into close contact for six years with an active group of biological workers, collaboration with whom was not only fruitful in immediate results but influenced the whole of his later work.

In 1909 Barger returned to academic work, being appointed in that year Head of the Department of Chemistry at Goldsmith's College in the University of London, and in 1913 he became Professor of Chemistry at the Royal Holloway College.

One of the first actions of the Medical Research Committee which was formed in 1914 was to collect its own team of research workers; Barger was appointed to be a member of the group and continued to hold this post during the period of the War.

In 1919 he was appointed to the newly constituted Chair of Chemistry in Relation to Medicine at Edinburgh and he held this position for eighteen years. A little more than a year before his death he became Regius Professor of Chemistry in the University of Glasgow; the latter important appointment was a fitting recognition of the distinguished position which he had attained in the profession of Chemistry in this country; it brought with it heavy burdens, but he threw himself into the arduous task of reorganizing a large department with

conspicuous freshness and enthusiasm; it is sad to think that the work with which he had made so much progress should have had to be left unfinished.

The aspect of Barger's scientific work which will be of major interest to readers of the *Biochemical Journal*, and for which indeed he will perhaps be chiefly remembered, is the group of researches which he made in the field of what he himself called the "simpler natural bases".

At the time when he took up his post in the Wellcome Laboratories the active principle of the suprarenal gland, adrenaline, had recently been isolated and its constitution had been determined. The chemistry of this substance naturally attracted much attention and Barger, in collaboration with H. A. D. Jowett, took up the attempt to synthesize it. In this he was not completely successful, but his efforts were embodied in an interesting paper (Barger & Jowett, *J. chem. Soc.* 1905, **87**, 967) on the synthesis of compounds closely related to adrenaline. These experiments led first to some interesting chemical work on the action of thionyl chloride on methylenedioxybenzenes (*J. chem. Soc.* 1908, **93**, 563); the much more important outcome of the experiments was, however, the interest which they aroused in Barger's mind in the physiological action of relatively simple basic compounds. This phase of his work, throughout which he collaborated with Dale, began with a return to the study of ergot of which he had already investigated the alkaloid content; his isolation of ergotoxine had done something to explain the physiological activity of extracts of ergot, but still left much unaccounted for; Barger first showed (*J. chem. Soc.* 1909, **95**, 1123) that a part of the unidentified activity was due to the presence of tyramine, which compound he also synthesized; later (Barger & Dale, *J. chem. Soc.* 1910, **97**, 2592), he isolated the still more active substance histamine from the same source.

In 1911 (Barger & Dale, *J. Physiol.* 1911, **41**, 499) Barger demonstrated for the first time the occurrence of histamine in an animal tissue (gut); later developments in physiology and pathology have given this observation an importance which could not at the time be appreciated. In connexion with this it may be mentioned that in continuation of Barger's work his pupil Ewins was subsequently able to isolate acetylcholine from a particular ergot extract, and this compound also has since proved to have a profound physiological importance. The physiological implications of one aspect of this work, as they were then apparent, were treated in the classical paper of Barger & Dale (*J. Physiol.* 1911, **41**, 19) in which the general conception of sympathomimetic amines was developed.

During this period Barger published several other papers dealing with compounds allied to those under discussion; among these may be mentioned the synthesis of hordenine, the alkaloid of barley (*J. chem. Soc.* 1909, **95**, 2193) and of hypaphorine, the betaine of tryptophan (Van Romburgh & Barger, *J. chem. Soc.* 1911, **99**, 2068), which occurs in the seeds of *Erythrina hypaphorus*.

With Ewins (*J. chem. Soc.* 1911, **99**, 2336) he showed that ergothioneine, a sulphur-containing substance which had been isolated by Tanret from ergot, was the betaine of thiolhistidine, whilst with Tutin (*Biochem. J.* 1918, **12**, 403) he was able to prove by synthesis that carnosine, which is a quantitatively important extractive of muscle, had the structure of β-alanylhistidine.

In later years Barger made another contribution in the field of amino-acid chemistry when he proved the constitution of the amino-acid methionine which had been discovered by Mueller in 1923. He synthesized this compound first in 1928 (Barger & Coyne, *Biochem. J.* 1928, **22**, 1417) and later by an improved method (Barger & Weichselbaum, *Biochem. J.* 1931, **25**, 997).

Of less immediate interest to biochemists, but of outstanding chemical importance, are the contributions which Barger made to the chemistry of

alkaloids: in his later years indeed work in this field occupied the greater part of his attention. His studies of alkaloids have been reviewed in detail in another place,[1] but brief mention may be made here of his more conspicuous achievements.

Barger's early study of the alkaloids of ergot, which was made in collaboration with F. H. Carr, has already been referred to. At the time when this study was begun considerable confusion existed concerning the physiologically active substances produced by ergot. The only crystalline alkaloid known to be extractable from the fungus was the ergotinine which had been discovered by Tanret, and this was physiologically inactive. Barger & Carr (*J. chem. Soc.* 1907, **91**, 307) described the isolation of a new alkaloid, $C_{35}H_{41}O_6N_5$, the salts of which could be obtained crystalline, and which showed a high degree of physiological activity. They showed, moreover, that the new alkaloid, which they named ergotoxine, was easily convertible into ergotinine and thus observed the first example of what has since turned out to be a general characteristic of all ergot alkaloids, namely that they occur in isomeric pairs, the members of which are interconvertible by simple means with large changes in specific rotation and physiological action.

Great physiological interest attaches to the work of Barger & Stedman (*J. chem. Soc.* 1923, **123**, 758; 1924, **125**, 1373; 1925, **127**, 247) on physostigmine (eserine); by skilful degradation experiments and close reasoning the structure of this alkaloid was completely elucidated. Not only was this work an important chemical achievement but it laid the foundation of the later well-known investigations of Stedman on the physiological action of simpler but analogous urethanes, among which at least one substance (the prostigmine prepared by Aeschlimann) has proved to be of great pharmacological and therapeutic value.

Two alkaloids which attracted Barger's early attention were carpaine and yohimbine. In the first case he published a useful preliminary paper (*J. chem. Soc.* 1910, **97**, 466) and more than twenty years later resumed work on the problem in collaboration with Robinson; in two further papers (Barger, Girardet & Robinson, *Helv. Chim. Acta*, 1933, **16**, 90; Barger, Robinson & Work, *J. chem. Soc.* 1937, p. 711) the constitution of the alkaloid was cleared up. The work on yohimbine consisted of a preliminary paper (Barger & Field, *J. chem. Soc.* 1915, **107**, 1025) on the degradation of the alkaloid, followed much later (Barger & Scholz, *J. chem. Soc.* 1933, p. 614) by the highly significant recognition of harman among the degradation products which immediately fixed the arrangement of three out of the five rings composing the structure; finally (Barger & Scholz, *Helv. Chim. Acta*, 1933, **16**, 1343) identification of further degradation products permitted the entire skeleton of the alkaloid to be formulated with tolerable certainty.

During the years 1928–33 Barger made a series of researches on a group of aporphine alkaloids, and with a succession of pupils, among whom may be mentioned Silberschmidt, Girardet, Eisenbrand and Schlittler, he was able to explain completely the constitutions of laurotetanine, pukateine, laureline and lauropukine.

In quite recent years he had been engaged with J. J. Blackie on a systematic study of the *Senecio* alkaloids; at the time of his death one of his main interests lay in the work which he was doing on the difficult problem of the constitution of calycanthine.

Reference should be made to one other piece of work which Barger did, not because it led to any results of importance, but because at one time it exercised

[1] Obituary Notice, *J. chem. Soc.* 1939, p. 715.

a peculiar fascination over his mind. It has already been mentioned that when he was in Brussels he studied a glucoside saponarin; this substance had the unusual property of giving a blue compound with iodine similar to that given by starch. The observation attracted Barger's interest and he returned to the subject on several occasions in later years; he found that the property of forming such blue complexes with iodine was generally associated with the γ-pyrone group, and he published several papers dealing with the theory of the reaction and with constitutional factors affecting it.

Finally, mention must be made of two researches in which Barger played a less direct but nevertheless important part, namely, the work of the present writer on thyroxine and that of A. R. Todd on vitamin B_1 (aneurin). At the time when the writer started to work on thyroxine he had already left Barger's laboratory several years. This did not, however, prevent consultation by correspondence, and the keenness of Barger's interest and the freedom with which he gave of his experience did much to further the progress of the work. It is typical of Barger that, in spite of this, he so far belittled the value of his own contribution as only with difficulty to be persuaded to agree to joint publication of the final stage.

The recent distinguished work of A. R. Todd and his collaborators on the constitution and synthesis of aneurin was begun and largely carried through in Barger's laboratory in Edinburgh. In the early stages of this work particularly, his contribution was of great value.

Apart from his original papers in scientific journals Barger published several books; in his monograph on *The Simpler Natural Bases* which appeared in 1914 he collected a large amount of chemical and physiological information which was otherwise not easily accessible. In 1930 he published *Some Applications of Organic Chemistry to Biology and Medicine*, and in 1932 a text-book entitled *Organic Chemistry for Medical Students*; the book by which he will chiefly be remembered, however, is the monograph *Ergot and Ergotism*, which appeared in 1931. The writing of this masterly book was a labour of love extending over many years; in it every aspect of the subject is treated with a sureness of touch and a scholarly finish which make the work a model of its kind.

Barger's work was recognized by many distinctions in this and other countries. He was elected a Fellow of King's College, Cambridge, in 1904. In 1919 he was admitted to the Fellowship of the Royal Society; he served on the Council in 1930–2, and a few weeks before his death he was awarded the Davy Medal. At the time of his death he was a Vice-President of the Chemical Society, of which in 1936 he was Longstaff Medallist. In 1934 he received the Hanbury Medal from the Pharmaceutical Society.

In 1928 he held the Baker visiting Professorship of Chemistry at Cornell University, and during the same year he delivered the Dohme lectures at the Johns Hopkins University, Baltimore. The British Association elected him President of Section B (Chemistry) for their meeting in South Africa in 1929.

He received honorary degrees from the Universities of Liverpool, Padua, Heidelberg, Michigan and Utrecht, and he was an honorary or corresponding member of many foreign academies.

Barger's bilingual upbringing was the foundation of the exceptional linguistic capacity which he developed in later life and which reached its climax when he was able to address the International Physiological Congress in Moscow in 1935 in eight different tongues. This facility for acquiring foreign languages was combined with a great enthusiasm for travel, and enabled him to make many contacts with colleagues in other countries. These contacts, which developed in

several cases into close friendships, were of great value not only to himself but to the cause of mutual understanding between scientific workers which he had so much at heart. He was truly recognized as an international figure in Science, and the many honours which he received from foreign Universities and Academies indicate the high regard in which he was held by his colleagues abroad; this regard was shown in another way, however, which meant even more to him, namely, by the constant flow of foreign students who passed through his laboratory.

Barger's death will be felt not only as a loss to science but as a personal grief to his many friends. Those who were but slightly acquainted with him may not easily realize to what an extent he possessed the gift of true friendship. He was not the most patient of men and his manner at times made him seem far less patient than he really was, so that the first stages of acquaintanceship with him were the most difficult and there were even some who never succeeded in surmounting the initial barrier. Those who were admitted to his friendship, however, found it difficult to remember that any such barrier had ever existed; there can, indeed, have been few men who were willing to do so much in any way and at any time for their friends, or who were capable of showing so true a human sympathy.

To his pupils he gave without stint, both of his thought to their problems, and of his time in the laboratory to their practical difficulties. At the same time he did all in his power to encourage independence of thought and was at pains never to dissuade a student from testing a new idea experimentally even though he might himself feel sure from his own experience that the reaction would not "go".

It was indeed this love for experiment which was the keynote of Barger's attitude towards his work; he was instinctively mistrustful of hypotheses which were allowed far to outrun experimental support and this habit of mind coloured his whole outlook. It caused him to have strong leanings towards a mechanistic philosophy, departure from which, as he said in a discussion on the nature of life at the meeting of the British Association in 1929, he could not but regard as treachery to Science.

He himself possessed a high degree of experimental skill and had been at particular pains to perfect himself in the technique of working with small amounts of material long before such technique came to be generally regarded as a part of the equipment of an expert organic chemist. This capacity for the fine handling of small quantities was somewhat paradoxically combined with unsystematic, not to say untidy, habits in ordinary laboratory work.

Barger remained always a fundamentally simple-minded man; completely honest and straightforward in his outlook he never hesitated to express his views on any subject on which he felt strongly. He had little patience with formality and little respect for conventions although he would not willingly disregard conventions in such a manner as to offend others who might feel differently. It was inevitable that he should at times have irritated less outspoken people, and thus have made things more difficult for himself, but he was not easily deterred by the thought of such consequences if what he had to say were a matter of conviction. This essential sincerity made it impossible for him effectively to conceal his true feelings, even on those occasions when he judged that such concealment would facilitate the attainment of his object.

Fundamentally non-political in outlook, Barger was a man of liberal views with whom it was a ruling desire to break down all barriers such as those of nationality which interfere with the free intercourse of men of science; enough

has already been said to indicate how great were his services in the pursuit of this idea, and in these times particularly the measure of success which he achieved remains an encouragement.

No account of Barger would be complete without reference to the warm hospitality of himself and Mrs Barger, not only to friends and colleagues, but to students who were working with him; for many who have passed through his laboratory the remembrance of this will be one of their happiest recollections.

Barger has died as he would have wished while still in full activity; he leaves to his friends the memory of a beloved personality whose loss is a grievous blow, and to his pupils an inspiration which will endure throughout their lives.

I am indebted to the Chemical Society for permission to reproduce here much material from the more comprehensive Obituary Notice which has already been published in their *Journal*.

C. R. Harington.

SERAFINO BELFANTI

1860–1939

Born at Castelletto Ticino (Italia Settentrionale) on 28 March 1860, Belfanti took his degree in medicine at the University of Turin in 1886, remaining there as assistant in the Institute of Physiological Chemistry and later in the General Medical Clinic, then under the direction of Camillo Bozzolo. Here in 1889 he carried out his first investigations, in the then rapidly expanding field of Bacteriology, on the adaptability of the tetanus bacillus to aerobic conditions; he also studied the polymorphic character of this organism, the diffusion of the diphtheria bacillus throughout the body and its localization in certain organs (broncho-pneumonia) whilst he drew attention also to the existence of healthy diphtheria carriers. In 1894 he took his doctorate in Bacteriology. About this time the serum treatment of diphtheria was introduced and made such a stir as to decide the Medical Association of Lombardy to found in Milan an institute for the preparation of antidiphtheritic serum. Belfanti was called to be the first director and from then onwards devoted his whole life to this enterprise. Under his firm and paternal guidance the Serotherapy Institute of Milan grew from its humble beginnings in a few small rooms to the vast building of to-day, a veritable workshop concerned with the production of most of the country's sera, vaccines, chemotherapeutic and biological agents. Owing to the personal enthusiasm and passion of Belfanti for investigation the Institute became not only a huge productive centre but a home for workers imbued with a love of scientific research. Belfanti's own publications, numbering about 150, extend into bacteriological, immunological and biochemical fields, and are characterized by originality, restraint and conciseness in exposition. Always there stands out the attitude of the biochemist. As early as 1898 with his assistant Tito Carbone he commenced a careful biochemical study of the nature of antitoxins, and after numerous experiments on the fractionation of antitoxic sera reached the conclusion that antitoxins in horse serum are associated with globulins precipitated by half saturation with ammonium sulphate. Time has brought numerous confirmations of this discovery. Again in collaboration with Carbone, Belfanti showed for the first time that red corpuscles could act as antigens, an observation which clarified the subject of haemolysis.

Towards the end of 1926, when he had passed his sixtieth year, he gave fresh proof of his passion for research by initiating with his pupils and the chemist Contardi a notable series of experiments on cytolytic products of degradation of phosphatides produced by the action of special ferments (lecithinases), and on the differentiation and classification of phosphatidases. Some of the latter results were published in the *Biochemical Journal*, organ of the Biochemical Society, to which Belfanti was proud to belong.

His attainments received widespread recognition. He was vice-president of the Reale Istituto Lombardo di scienze e lettere, member of the executive of the Consiglio Nazionale delle Ricerche (National Research Council) and of many academies and scientific societies in Italy and abroad, including the Deutsche Akademie der Naturforscher, the Biochemical Society and the Indian Academy of Science. In 1934 he was nominated Senator of the Kingdom of Italy. In 1929 he founded the Italian section of the International Society of Microbiology with the object of assisting Italian bacteriology and aiding in its diffusion abroad.

The name of Serafino Belfanti will remain indissolubly bound to the growth of Bacteriology and Immunity. His faith in work, his cordiality and simplicity of character, his frank and friendly smile and lavish hospitality will long remain as precious memories to his pupils, friends and colleagues.

On 6 March 1939, after a brief illness, Belfanti's illustrious and fruitful career came to an end. Up to the last he interested himself in the work of his Institute, suggesting experiments and stimulating ideas until a few days before his death. He was buried in the family tomb at Castelletto Ticino, the land of his birth which he loved so well.

CVI. THE GLYCOLYTIC ACTIVITY OF BRAIN

I. MACERATED BRAIN TISSUE

By ALEXANDER GEIGER and JONATHAN MAGNES

II. EFFECT OF CYTOLYSIS, THE PRESENCE OF A GLYCOLYTIC INHIBITOR

By ALEXANDER GEIGER

From the Physiological Laboratory, the Hebrew University, Jerusalem

(*Received 6 February 1939*)

I. MACERATED BRAIN TISSUE

Knowledge of brain glycolysis compares unfavourably with that available for muscle. As yet, none of the intermediary steps of brain glycolysis is known with certainty and our knowledge of the coenzymes involved in brain glycolysis is very incomplete. In view of the conflicting statements in this field, we have here investigated the effects on brain glycolysis of general experimental conditions, of coenzymes and of certain of the known intermediary products of muscle glycolysis. It will be shown here that none of the substances which are intermediary products of muscle glycolysis can be regarded as such in glycolysis with intact brain cells. No alternate indication, however, of the intermediary steps involved in brain glycolysis can be given on the basis of these experiments.

Since finishing this work, cell-free extracts of brain with high glycolytic activity have been obtained. The results obtained with extracts—which differ somewhat from those obtained with intact cells—will be described in a later paper. Consequently, we wish to emphasize that when the present work was done, only intact brain cells were found to be glycolytically active; therefore the conclusions reached in this paper are valid for intact brain cells only.

Methods. The technique used was mainly that described previously [Geiger, 1935]. All the experiments were carried out on 2–6 months old albino rats, unless otherwise stated. Glucose was determined by Shaffer & Somogyi's method [1933]. Later the more recent modification [1937] of this method was used. Cozymase was prepared by Euler's method and later according to Meyerhof & Ohlmeyer [1937]. The adenosinetriphosphate[1] was prepared according to Barrenscheen & Filz [1932]. Warburg manometers were used for the measurement of respiration. Lactic acid was determined by the method of Friedemann & Graeser [1933].

The effect of rat age and the season on brain glycolysis. Macerated brain cortex of young rats shows a higher glycolytic rate than that of old rats. Table I contains a set of parallel experiments with macerated brain cortex from rats 2–3 months old and from rats more than a year old. In all the experiments, a measured amount of macerated brain was suspended in a known amount of

[1] Henceforth adenosinetriphosphate will be abbreviated AP, creatinephosphate CRP, reduced glutathione GSH, and oxidized glutathione GSSG.

M/15 phosphate buffer containing 200 mg. per 100 ml. of glucose, 50 mg. per 100 ml. of adenosine triphosphate and 50 mg. per 100 ml. of glutathione, all at *p*H 7·1.

Table I

Dilution of the tissue macerate	mg. lactic acid formed by 100 g. brain per hr.	
	Old rats	Young rats
1 : 100	340	510
2 : 100	340	530
3 : 100	338	480
4 : 100	330	435
5 : 100	320	410
6 : 100	300	375

The seasonal variation in glycolytic activity is also noteworthy. Higher values were obtained with our rats during the months of July and August. 120 determinations, under otherwise identical conditions, showed an average lactic acid production of 465 mg. per 100 g. tissue per hr. in winter and spring, as compared with 613 mg. during the late summer months.

The effect of dilution of the tissue. As Table I shows, dilution increases the relative glycolytic power of macerated brain cortex. In these experiments only the concentration of the tissue was varied.

We were also able to confirm Ashford's [1934] observation that tissue slices possess a much higher glycolytic power than macerated tissue.

The effect of the composition of the medium. It was observed by Ashford [1934] that the rate of brain glycolysis is lower in phosphate buffer than in bicarbonate Ringer. We are able to confirm this. We found, however, that if AP is added to macerated brain cortex in phosphate buffer, the glycolysis reaches almost the same level as it does in bicarbonate Ringer with added AP. This is illustrated in Table II.

Table II

Reaction-media: all at *p*H 7·2	mg. lactic acid formed by 100 g. tissue in 1 hr.
M/15 phosphate buffer	370
M/15 phosphate buffer and AP	550
Bicarbonate Ringer	560
Bicarbonate Ringer and AP	590

Glycolysis with various brain cortex preparations. In previous experiments [Geiger, 1935] extracts of brain cortex were made which had very little glucolytic activity in comparison with that of cortex macerate. Ashford [1934] was unable to obtain active extracts of brain cortex. Mazza & Malaguzzi-Valeri [1935] as well as Euler *et al.* [1936], however, described experiments with brain extracts showing much greater glucolytic activity.

In the present experiments we were unable to obtain extracts more active than those described previously. The NaCl or KCl extracts, even after addition of activators, had only about 1/10 of the activity of the brain cortex from which they were prepared. It should be noted, moreover, that only the first extract of macerated brain is at all active. Further washings on the centrifuge with isotonic KCl or NaCl did not yield active extracts and did not impair the glycolytic activity of the brain macerate. Washing with water destroyed the activity of the macerate.

In the experiments presented in Table III, ice-cooled brain cortex was carefully macerated in an ice-cold solution of NaCl or KCl. The suspension was

centrifuged and the residue washed several times more in the centrifuge. The first extract or the first and second extracts combined were made up with buffer solution to the same volume as the residue. In this way corresponding amounts of diluted extract and of the washed tissue suspension were obtained for the experiments.

Table III

	mg. lactic acid formed by 100 g. tissue per hr.	
Weight of tissue, g.	0·35	0·425
Unwashed + AP	444	—
Washed, no addition	—	242*
Washed + AP + GSH	382†	426*
Extract corresponding to same amount of tissue	—	6
First extract + AP + GSH	41	15
Tissue and extract + AP	421	—

* Washed thrice. † Washed once.

In later experiments it was observed that the destruction of the cell structure by distilled water, or by macerating with finely ground quartz-sand, completely destroyed the glycolytic activity of the brain. These experiments will be described in a later paper.

Lactic acid formation from glycogen. In Haarmann's [1932] and also in Ashford's experiments, the amount of lactic acid formed by macerated brain from glycogen was small as compared with that from glucose. Euler *et al.* [1936], however, obtained with brain extracts lactic acid formation from glycogen which sometimes equalled that obtained from glucose.

We repeated these experiments using both brain macerate and brain extracts, on the supposition that these conflicting results could be explained by the difference in the enzymic materials of these two preparations. Table IV shows the results.

Table IV

	mg. lactic acid formed by 100 g. brain cortex per hr.
Macerated brain suspension + AP + GSH + glucose	612
Macerated brain suspension + AP + GSH + glycogen	36
Macerated and washed twice + AP + GSH + glucose	589
Macerated and washed twice + AP + GSH + glycogen	31
Brain extract + AP + GSH + glucose	45
Brain extract + AP + GSH + glycogen	32

These experiments confirm the findings of Ashford [1933] according to which the amounts of lactic acid formed by macerated brain cortex from glycogen are small, as compared with the amounts formed from glucose.

In the extracts, the lactic acid formation from glycogen is as low as that in the macerated tissue, but since the glucose-splitting activity of the extracts is much lower than that of the macerated tissue, the relative rates of these two processes in the extract are different from those in the macerated tissue. In other words, the seemingly different behaviour of the extract and the macerate towards glycogen is merely due to the fact that under these conditions only a very small fraction of the glucose-splitting enzyme of the brain is active in the extract, while the activity of the glycogenolytic enzyme system is the same in the extract as in the original tissue.

Wilstätter & Rohdewald [1937] found that leucocytes and yeast transform glucose into glycogen prior to the formation of hexosediphosphate. In similar experiments made with brain no glycogen formation from glucose could be observed.

The inability of macerated brain cortex to form lactic acid from added hexosediphosphate. It was shown by Ashford [1933] and by Edlbacher *et al.* [1934] that brain cortex is able to produce small amounts of lactic acid from hexosediphosphate. Upon repeating these experiments with varying amounts of hexosediphosphate we found that the more hexosediphosphate added to chopped brain cortex, the greater the lactic acid formation. These experiments are contained in Table V.

Table V

	mg. lactic acid formed per hr. by 100 g. tissue	
	Exp. 1	Exp. 2
5 mg. hexosediphosphate at once	57	—
5 mg. hexosediphosphate after 1 hr.	62	—
10 mg. hexosediphosphate at once	100	121
10 mg. hexosediphosphate after 1 hr.	180	172
30 mg. hexosediphosphate at once	150	169
30 mg. hexosediphosphate after 1 hr.	380	376
60 mg. hexosediphosphate at once	—	250
60 mg. hexosediphosphate after 1 hr.	—	565

The amounts of hexosediphosphate indicated in this table were added to 10 ml. of brain suspension containing 0·4 g. of chopped brain cortex.

This singular behaviour of hexosediphosphate can be explained on the basis of the enzymic equilibrium between hexosediphosphate and dihydroxyacetonephosphate, discovered by Meyerhof & Lohmann [1934]. According to these authors zymohexase is present in brain in much smaller quantities than in muscle. When hexosediphosphate is added to the brain macerate, triosephosphate is formed by the action of zymohexase in amounts depending—*ceteris paribus*—on the amounts of hexosediphosphate present. The rate of the process is proportional to the amount of enzyme present. The triosephosphate is in turn transformed into lactic acid artificially during the precipitation of the carbohydrates by copper-lime. This is illustrated in Table VI. Lactic acid and triosephosphate determinations were carried out immediately and at various time intervals after the addition of a known amount of hexosediphosphate to the brain pulp suspension. The triosephosphate was determined in the trichloroacetic filtrates according to Meyerhof & Lohmann [1934] by determining the amount of P liberated in 2*N* alkali at room temperature in 20 min.

Table VI

		mg. lactic acid formed	mg. triosephosphate (P_2O_5)
With hexosediphosphate:	At once	0·420	0·537
	7 min. later	1·680	1·740
	15 min. later	1·680	1·730
	30 min. later	1·600	1·700
	60 min. later	1·548	1·680

From these experiments it is evident that 7 min. after the addition of hexosediphosphate a certain amount of lactic acid is already "formed" and that this amount does not increase with time. On the other hand, estimation of triosephosphate shows it to be present in amounts corresponding to the amounts

of lactic acid found after treatment with copper-lime. It can therefore be safely deduced that chopped brain forms no lactic acid enzymically from hexose-phosphate and that the amount of lactic acid found in the experiments is derived from the artificial transformation of the triosephosphate which in turn is formed from hexosediphosphate by zymohexase. The relatively long time necessary for establishing the equilibrium between hexosediphosphate and triosephosphate in the brain pulp is due to the low zymohexase content of the brain. According to Meyerhof & Lohmann [1934] the zymohexase content of brain is only 1·6 % of that in voluntary muscle, and in our experiments this small amount is diluted about 30 times. From the same experiments it may therefore be concluded that the brain is unable to form lactic acid from triosephosphate.

The inability of brain to convert hexosediphosphate into lactic acid led us to consider the possibility that lactic acid formation from glycogen by the brain is also effected via glucose. In this case the rate of lactic acid formation should depend on the diastatic activity of the brain. As the experiments in Table VII show, the addition of diastase to the brain pulp greatly increases the lactic acid formation from glycogen.

Table VII

	mg. lactic acid formed by 100 g. tissue per hr.	
	Exp. 1	Exp 2
Brain pulp + glucose	557	472
Brain pulp + glucose + diastase	573	481
Brain pulp + glycogen	13	11
Brain pulp + glycogen + diastase	153	287

In these experiments 0·4 g. of brain pulp was suspended in 10 ml. of Ringer's solution. AP and glutathione were added to each flask.

Autolysis of brain cortex. In the hope of obtaining a suitable brain cortex preparation for studying the activators of brain glycolysis—brain extracts not being suitably active—we tried to eliminate the coenzymes, at least in part, by autolysing brain cortex in the absence of glucose. A series of experiments were made under different conditions, the results of which are summarized in Table VIII.

These experiments were made by macerating the brain in an isotonic NaCl solution, the required *p*H being obtained by the addition of HCl or $NaHCO_3$. This suspension was shaken in a water bath at 37° for a known time in an atmosphere of N_2, O_2 or air. Subsequently, equal amounts of the neutralized brain suspension were transferred in a measured amount of bicarbonate-Ringer to the experimental flasks containing glucose and the necessary additions. The flasks were then incubated at 37° for 1 hr.

Table VIII

	mg. lactic acid formed by 100 g. tissue per hr.			
	E. 105		E. 108	
	Glucose	Glucose + AP + GSH	Glucose	Glucose + AP + GSH
Before incubation	383	434	—	388
Incubated in N_2 for 1 hr. at neutrality	137	289	56	247
Incubated in O_2 for 1 hr. at neutrality	149	328	143	326

As Table VIII shows, autolysis in N_2 inactivates more completely than in O_2, and brain macerate inactivated in O_2 can be reactivated more completely by the addition of AP and GSH than when inactivated in N_2.

The rate of inactivation at different reactions was investigated in similar fashion. These experiments show that at an alkaline reaction the brain suspension becomes very rapidly inactivated and cannot be reactivated by the addition of AP and GSH. In an acid reaction the inactivation proceeds more quickly than at neutrality but reactivation can still be effected if the suspension is not autolysed for too long a time. In this connexion it is interesting to note that according to Giri & Datta [1936] brain phosphatases are inactive at neutrality and exhibit their greatest activity in the alkaline and acid ranges at about *p*H 5·0 and 9·0. In experiments during the late summer months we were unable to obtain as complete an inactivation by autolysis in O_2 at neutrality, even when we extended the time of autolysis to 2 hr. At the same season the rate of glycolysis of the unautolysed tissue was found to be much higher than in the experiments made in the winter, spring and early summer months.

Activators of brain glycolysis

Adenosinetriphosphate was found to activate brain glycolysis. The experiments in Table IX show the rate of glycolysis with macerated brain cortex with and without the addition of AP.

Table IX

	mg. lactic acid formed by 100 g. tissue per hr.	
*p*H	Glucose only	Glucose + AP
6·00	140	145
6·40	325	342
7·00	441	533
7·50	389	580
8·00	279	555

At neutrality, the addition of about 3 mg. of AP to 10 ml. of brain suspension was sufficient for maximum activation. In the acid range, AP has practically no activating effect. Maximum *activation* occurs in the alkaline range. The maximum *rate* of glycolysis, however, is found at neutrality. In these experiments the fresh brain cortex of rats was used without previous autolysis, so that the brain itself contained some AP. The experiments were made in phosphate buffer, in which, as previously shown, the original amount of AP in the brain is not sufficient to cause full activation.

In order to be the better able to test the activating effect of several substances known to be present in brain macerate, we autolysed the fresh macerate at neutrality in air for 1 hr. at 37° as previously described. Table X contains

Table X

	mg. lactic acid formed by 100 g. tissue per hr.	
	Exp. 1	Exp. 7
Before autolysis + glucose + AP + GSH	482	486
Autolysed brain cortex + glucose	34	92
Autolysed + glucose + AP	223	410
Autolysed + glucose + CRP	40	100
Autolysed + glucose + hexosediphosphate	162	226
Autolysed + adenylic acid + CRP + glucose	—	95
Autolysed + glucose + AP + hexosediphosphate + CRP	289	421
Autolysed + cozymase + glucose	62	90

experiments of this type made with a suspension of macerated and autolysed brain cortex in bicarbonate Ringer.

To calculate the degree of activation in those experiments in which hexosediphosphate was also added, we subtracted the amount of lactic acid found in the flasks to which only hexosediphosphate was added, from the lactic acid content of the other flasks, which contained hexosediphosphate and other additions.

These experiments show that AP is able to bring about a maximal activation when added alone. None of the other substances caused the same degree of activation provided that the inactivation of the brain suspension was complete enough. The activating effect of hexosediphosphate seems to depend on the degree of inactivation of the tissue. The greater the inactivation of the brain suspension the less the activating effect of hexosediphosphate alone. After a complete autolysis in N_2 the brain pulp could not be reactivated by AP. Cozymase had no activating effect on brain glycolysis.

Table XI

	mg. lactic acid formed by 100 g. tissue per hr.			
	Fresh chopped brain in phosphate buffer		Autolysed brain	
Additions to brain pulp	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Glucose only	460	440	74	90
Glucose + AP	574	700	253	312
Glucose + hexosediphosphate	618	597	126	202
Glucose + AP + hexosediphosphate	618	—	260	307
Glucose + adenylic acid	465	427	68	95
Glucose + adenylic acid + hexosediphosphate	618	707	255	318

The experiments in the foregoing table show that adenylic acid alone has no activating effect but that the combination of adenylic acid and hexosediphosphate gives as high an activation as the addition of AP. Hexosediphosphate alone may sometimes also give full activation. This is probably due to the rapid dephosphorylation of the brain AP and the slower process of deamination of the adenylic acid.

Transference of phosphorus to adenylic acid

According to the findings of Parnas and co-workers, added hexosediphosphate is broken down in muscle to phosphopyruvic acid which transmits its phosphorus to the adenylic acid.

The following experiments show that in the brain this mechanism is different. Phosphopyruvic, added together with adenylic acid, is unable to activate glycolysis.

Table XII

	mg. lactic acid formed by 100 g. brain per hr.		
	Not autolysed	Autolysed brain	Autolysed brain
Glucose without addition	460	76	132
Glucose and adenylic acid	495	69	126
Glucose and adenylic acid and hexosediphosphate	618	397	402
Glucose and adenylic acid and phosphopyruvic acid	512	79	138
Glucose and adenylic acid and phosphoglyceric acid	—	83	140
Glucose + adenosinetriphosphate	—	382	398

Another proof that a brain suspension is unable to split phosphopyruvic acid is given by experiments in which the activating effects of small amounts of pyruvic acid and phosphopyruvic acid are compared. According to Mendel *et al.* [1931] small amounts of pyruvic acid (9–40 mg. per l.) added to brain cause an increased lactic acid production. The experiments in Table XIII show that the addition of phosphopyruvic acid is unable to bring about this activation with brain, although pyruvic acid does.

Table XIII

	mg. lactic acid formed from 100 g. brain per hr.		
	E. 145	E. 142	E. 163
Glucose and AP	495	—	427
Glucose and AP and pyruvic acid	615	825	759
Glucose and AP and phosphopyruvic acid	512	555	481

These experiments indicate that the transference of phosphate to adenylic acid, if such a mechanism exists in brain, is not via phosphopyruvic acid but by some other route. It has been shown by Neuberg [1935], by Lutwak-Mann & Mann [1935] and by Ohlmeyer [1935] that in yeast the direct transfer of P from hexosediphosphate to adenylic acid is possible. The experiments just described suggest the existence of this mechanism in brain.

Phosphorylation

A further feature of brain glycolysis is the apparent absence of phosphoric ester formation. Ashford & Holmes [1929] and others were unable to demonstrate any phosphorylation. Euler *et al.* [1936], however, report experiments on brain extracts in which a disappearance of inorganic phosphate during glycolysis was observed.

In view of the strong activating effect of AP on brain glycolysis and of its role in muscle glycolysis as a phosphate carrier, it was felt necessary to reinvestigate this question. In our experiments made with and without the addition of AP and NaF, no phosphoric ester formation could be demonstrated. Moreover, in accordance with Ashford & Holmes' [1929] findings, a slight liberation of inorganic phosphate was found.

The role of glutathione

In a previous paper [Geiger, 1935] experiments in which GSH had a marked activating effect on brain glycolysis were described. A similar effect had been observed by Quastel & Wheatley [1932] in yeast fermentation. Euler *et al.* [1936] confirmed the observation that GSH had a slight activating effect on lactic acid production by brain extract. These authors attribute the activating effect of GSH to its capacity for combining with heavy metals.

A large number of experiments carried out by us during the years 1935–6 have shown that the activating effect of GSH—although a constant factor in every experiment—is of a very different order of magnitude in different experiments. It may happen that in two experiments which are identical in every respect, except for the rats, the activating effect of GSH is as high as 100% in the one and not more than 10% in the other. Table XIV contains experiments selected at random from a large number of experiments made under different conditions.

Table XIV

	mg. lactic acid formed							
	Exp. A		Exp. B		Exp. C		Exp. D	
	No GSH	GSH added	No GSH	GSH added	No GSH	GSH added	No GSH	GSH added
Brain cortex of young rats in bicarbonate-Ringer	410	500	487	512	330	462	243	533
Brain cortex of old rats in bicarbonate-Ringer	320	426	229	346	274	306	222	451
Brain cortex in phosphate buffer *p*H 7·2	245	355	270	290	248	296	361	381
Brain cortex in phosphate buffer with added AP	385	492	399	411	367	416	239	500
Washed twice with 0·9 % NaCl, then AP added	396	466	443	470	339	510	470	485

The only factor influencing the activating effect of GSH was the H^+ concentration of the medium. The activating effect of GSH is largest as a rule at *p*H 6·4 and diminishes with increasing alkalinity. The behaviour of GSH towards *p*H is thus directly opposite to that of AP which activates more strongly on the alkaline side.

Nutrition is another factor which has some influence on the activating effect of GSH. The addition of GSH to the brain pulp of starved or vitamin B_1-deficient rats produces a more marked activation.

The possibility that the activating action of GSH was due to its binding the traces of Cu or Fe present in the brain pulp (or possibly in the reagents used) may be discarded on the basis of the relatively large amounts of Cu which are necessary to cause inhibition. In a series of experiments various amounts of Cu and Fe were added to the experimental flasks. Table XV shows that the addition of 0·04 mg. $CuSO_4$ to 10 ml. brain suspension causes only a very slight inhibition of brain glycolysis. It is remarkable what large amounts of Cu are necessary to cause inhibition in brain macerate, as compared with muscle glycolysis. These experiments showing that small traces of heavy metals have no effect on brain glycolysis exclude the possibility that the activating effect of GSH on brain glycolysis is due to the binding of traces of Cu or Fe originally present in the macerate as suggested by Euler *et al.* [1936].

Table XV

	mg. lactic acid formed by 100 g. brain per hr.	
	$CuSO_4$	$FeCl_3$
No addition	565	581
With 0·40 mg. % heavy metal salt	520	—
With 1·00 mg. % heavy metal salt	460	567
With 2·00 mg. % heavy metal salt	470	—
With 20·00 mg. % heavy metal salt	410	—

The effect of oxidized glutathione

The statement was made by one of us [Geiger, 1935] that oxidized glutathione strongly inhibits muscle glycolysis and also inhibits brain glycolysis to a smaller extent. It appears from later experiments that this statement was erroneous. The error was caused by the presence of a relatively large amount of Cu in the glutathione preparation used. Later it was shown by Wagner-Jauregg & Rzeppa [1936] that minute amounts of Cu inhibit muscle glycolysis. It was also observed

later by us that the Cu liberated from our glutathione preparation on oxidation was sufficient to cause an almost complete inhibition of muscle glycolysis. When benzoin-oxime was added to precipitate the Cu liberated by oxidation, no inhibitory effect was observed. A recrystallized preparation of glutathione or a commercial one did not inhibit, or at most only very slightly when employed in large quantities. Table XVI shows the results of these experiments. (Benzoin-oxime, when added to the muscle preparation, had no effect on glycolysis.)

Table XVI

	mg. lactic acid formed by 100 ml. muscle extract in 1 hr.
50 mg. % GSSG containing Cu	181
50 mg. % GSSG containing Cu + benzoin-oxime	1120
50 mg. % recrystallized GSH oxidized	1210
50 mg. % commercial GSH oxidized	1220
Without GSSG	1180

Discussion

The experiments described in this paper show that the glycolytic process in the brain differs markedly from that in muscle. One difference is that cytolysis of brain cortex with distilled water causes complete inhibition of glycolysis. Another is the difference in the behaviour of the two systems towards inhibitors such as fluoride, iodoacetate or oxalate. All these substances inhibit both brain and muscle glycolyses. However, in the case of brain, when any of these substances is present, no phosphorylated intermediary products are formed, and glucose is not attacked at all. No phosphoric ester formation can be demonstrated in the course of brain glycolysis.

Copper ions, which according to Wagner-Jauregg & Rzeppa [1936] completely inhibit muscle glycolysis in concentrations of 10^{-5} *M*, do not inhibit brain glycolysis in concentrations up to 10^{-3} *M*. In this connexion Bauer's findings [1936], according to which Cu^{++} inhibits the action of zymohexase, may be of interest. Since, as has been shown in this work, brain cannot form lactic acid from hexosediphosphate or triosephosphate, the selective inhibition of the action of zymohexase would not inhibit brain glycolysis.

There is definite proof that the way of breakdown of glucose and the intermediary products involved depart in many respects from the scheme set up for muscle glycolysis. The present paper shows that no lactic acid can be formed in brain from added hexosediphosphate, phosphoglyceric acid or phosphopyruvic acid. Brain is unable even to hydrolyse phosphopyruvic acid. It was demonstrated by Johnson [1936] that α-glycerophosphate and pyruvic acid cannot be regarded in brain as possible precursors of lactic acid.

It may be added that the absence of lactic acid formation in brain from added zymophosphates cannot yet be regarded as a conclusive proof against phosphorylation. As has been shown here, only intact cells are able to form lactic acid from glucose. Cytolysed brain suspensions, as will be shown in a later paper, are unable to attack glucose. It is therefore possible that the problems presented by living yeast—in which case phosphorylation is still under discussion—also apply to brain. Dickens [1936] has claimed that the entry of glucose into brain cells is accompanied by phosphorylation.

On the other hand, recent work by Needham & Lehmann [1937] and Needham & Nowiński [1937] has shown that hexosediphosphate does enter into embryonic cells, although practically no lactic acid is formed from it. The work of Needham

and his collaborators shows in many respects a striking similarity between embryonic and brain glycolyses.

It is however difficult to explain the activating effect of adenosinetriphosphate in a non-phosphorylating glycolysis. All our experiments show the absence of phosphorylation and the inability of brain to form lactic acid from phosphorylated compounds. On the other hand, adenosinetriphosphate which is considered as a transporter of P, activates the glycolysis in inactivated brain in some cases by as much as 300–400 %.

Summary

1. The glycolytic rate of brain from adult rats is lower than from that of young animals.

2. The glycolytic rate in brain pulp increases with increased dilution of the tissue by isotonic saline solutions.

3. Higher glycolytic rates were obtained in Ringer's solution than in a phosphate buffer solution of corresponding *p*H. If adenosinetriphosphate is added to the phosphate solutions the glycolytic rate attains that reached in Ringer's solution.

4. Extraction with water destroys the glycolytic activity of brain. Repeated washing of brain pulp with isotonic solutions does not diminish its glycolytic power.

5. Only small amounts of lactic acid are formed by brain from glycogen. Brain extracts possess the same glycogenolytic activity as brain tissue. Owing to the low glycolytic activity of these extracts, the relationship—glycogenolysis: glucolysis is different in the extracts from what it is in the tissue.

6. No lactic acid is formed from hexosediphosphate by brain. The apparent lactic acid formation from hexosediphosphate is due to the slow formation of triosephosphate, which in turn is transformed into lactic acid during the analysis.

7. Brain pulp when incubated at 37° in N_2 or O_2 without addition of glucose becomes partially inactivated. The glycolytic power of the autolysed pulp may be partly restored by adenosinetriphosphate and glutathione. Adenosinetriphosphate may be replaced by a combination of adenylic acid and hexosediphosphate. Brain pulp inactivated at *p*H 8·0 cannot be reactivated by these substances.

8. Adenosinetriphosphate may be regarded as a coenzyme of brain glycolysis. Under the present experimental conditions, cozymase and creatinephosphate have proved to be ineffective.

9. It is indicated that P may be transferred by brain from hexosediphosphate to adenylic acid but not from phosphopyruvic acid.

10. Brain tissue is unable to dephosphorylate phosphopyruvic acid.

11. No phosphoric ester formation from inorganic phosphate can be demonstrated with intact brain cells.

12. The activating effect of glutathione on brain glycolysis is very irregular. It is however demonstrated that this activating effect is not connected with the presence of heavy metals.

13. A former statement, according to which oxidized glutathione inhibits glycolysis, was erroneous. The inhibition in these experiments was due to the presence of small amounts of Cu in the glutathione preparation.

14. It is suggested that the way of breakdown of glucose in brain departs in every respect from the way of breakdown of glycogen in muscle.

II. EFFECT OF CYTOLYSIS, THE PRESENCE OF A GLYCOLYTIC INHIBITOR

In the first part of this paper it was reported that brain cortex loses all its glycolytic power if suspended in distilled water or if ground thoroughly with quartz sand. Case [1929] found that only intact brain cells are able to activate lactic acid formation from glucose by muscle extract. Dickens [1936] found that, following destruction of brain cells by grinding with sand, glucose oxidation ceases. Similar observations have been reported by Jowett & Quastel [1937].

It appears, however, that the necessity of intact cell structure for glucose breakdown is not limited to the brain cells. Irving [1927] stated that the integrity of kidney cells is essential for the utilization of glucose. The same view was also held by Case [1929]. The glycolytic activity of tumour cells is completely destroyed by freezing, grinding and by extracting with water according to Barr *et al.* [1928]. The same observation was made by the author on rat uterus (unpublished). According to Needham & Nowiński [1937] the destruction of cell structure abolishes glycolysis in the embryo.

Young [1929], Penrose & Quastel [1930] and Yudkin [1937] showed that cytolysis of certain bacteria results in the destruction of glucose dehydrogenase and of lactic dehydrogenase.

Brain extracts retain only a very small fraction of the glycolytic activity of the original tissue, the bulk of the activity remains in the tissue, and is not removed by further extractions [Part I]. Extraction with distilled water completely destroyed the glycolytic activity in the brain tissue.

Boyland *et al.* [1937] recently succeeded in activating a cell-free extract from "Crocker 180" tumour by adding relatively large amounts of cozymase and adenylic acid. These extracts produced lactic acid from glucose as rapidly as did the original tissue. These authors believe that tumour extracts during extraction are inactivated by nucleosidases liberated on destruction of the tumour cells. It appears therefore that the lack of glycolytic activity in tumour cell extracts is due to the destruction of coenzymes.

The earlier part of the present experiments was undertaken to ascertain the effects of cytolysis of brain tissue on the glucose breakdown and to study the nature of the inhibition. In the later part an inhibitor of glycolysis which is liberated from the brain on cytolysis is described.

The effect of suspension in water, of grinding with sand and of freezing, on the glycolytic power of brain cortex

Brain cortex ground in a smooth-surfaced mortar and suspended in isotonic NaCl solution has only slightly lower glycolytic activity than slices prepared from the same brain. If, however, the brain is ground with the addition of fine quartz sand the activity of the resulting suspension decreases considerably, the decrease in activity depending on the thoroughness of grinding. A complete loss of activity results from suspending the brain pulp in distilled water or from repeated freezing and thawing of the brain pulp [Table I]. For these experiments rat brain hemispheres were hashed with scissors. Aliquot parts of the hash were treated in the manner indicated in Table I and were then suspended in four parts of water or in isotonic NaCl solution. After standing at room temperature for

10 min. the suspensions were distributed in Erlenmeyer flasks containing bicarbonate-Ringer, glucose, glutathione and AP. Lactic acid was determined chemically.

Table I

	mg. lactic acid formed by 100 g. brain in 1 hr.
Ground in smooth mortar with 0·9% NaCl	712
Ground with sand with 0·9% NaCl	384
Thoroughly ground with sand with 0·9% NaCl	95
Frozen and thawed twice with 0·9% NaCl	64
Ground in smooth mortar, suspended in distilled water	35
Ground in smooth mortar, suspended in 4% NaCl	375
Ground in smooth mortar, suspended in 12% sucrose	621
1 : 5 0·9% NaCl extract of brain	67
1 : 5 water extract of brain	4

In these experiments the amount of glucose which disappeared roughly corresponded to the amount of lactic acid formed. There was no phosphoric ester formation, but liberation of some inorganic phosphate was observed. It is to be noted, that, while the extracts prepared with istonic solutions possessed some activity, no glycolysis at all was observed in the water extracts.

Dickens [1936] stated that after the destruction of brain cells by grinding with sand, Robison ester could still be oxidized, although glucose oxidation was no longer appreciable. Therefore in the following experiments (Table II) several substances were tried as glycolytic substrates in cytolysed brain. The experiments were made in the same way as those in Table I. One of these experiments is contained in Table II.

Table II

	mg. lactic acid by 100 g. in 1 hr.	ml. O_2 consumed by 100 g. in 1 hr.
Cytolysed brain with glucose	71	36
Intact brain pulp with glucose	680	720
Cytolysed brain with glycogen	43	—
Intact brain pulp with glycogen	39	—
Cytolysed brain with Robison ester	14	11
Intact brain with Robison ester	16	—
Cytolysed brain with hexosediphosphate*	0	0
Intact brain pulp with hexosediphosphate	0	0
Cytolysed brain with α-glycerophosphate and pyruvic acid	33	—
Cytolysed brain with lactate	—	46
Intact brain pulp with lactate	—	745
Cytolysed brain with succinate	—	900
Intact brain pulp with succinate	—	1095

* The initial values were taken after 10 min. incubation at 37°.

The respiration of brain is greatly restricted on cytolysis. The O_2 uptake with glucose and with lactic acid is very small. No more O_2 was taken up with Robison ester than with glucose; the oxidation of succinic acid however was practically unaffected by cytolysis. The glyoxalase activity of brain was also unimpaired after cytolysis. The glycolytic activity of cytolysed brain could not be restored by the addition of adenosinetriphosphate and of cozymase. No appreciable decoloration of methylene blue could be observed with cytolysed brain in the presence of AP, or adenylic acid or cozymase. Boyland & Boyland [1935] stated that tumour extracts oxidatively deaminate adenosinetriphosphate and cozymase.

The presence of a glycolysis inhibitor in cytolysed brain

Cytolysed brain cells or an alkaline brain extract inhibit the glycolysis of muscle extracts and the fermentation of yeast extracts. This is the more remarkable since Case [1929] found that the addition of intact brain cells to muscle extracts enabled the latter to form lactic acid from glucose.

It was found by Winfield & Hopkins [1915] that pancreas contains a substance which inhibits lactic acid formation from glycogen in muscle. It was later demonstrated by Case & McCullagh [1928] that this substance is amylase. The pancreatic inhibitor did not inhibit lactic acid formation from hexosediphosphate, or from glucose when hexokinase was added to the muscle extract. The same observation was made by Barr *et al.* [1928]. As the following experiments show, the antiglycolytic substance in brain is not amylase although it is probably an enzyme.

The inhibition by cytolysed brain of lactic acid formation in muscle extract from different substances

Preliminary experiments have shown that small amounts of fresh brain pulp suspended in distilled water, when added to muscle extract, inhibit lactic acid formation from glycogen. Extracts of cytolysed brain, made with distilled water,

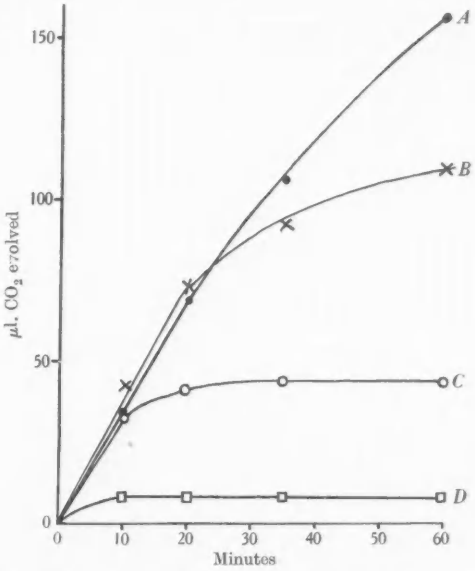

Fig. 1. The effect of cytolysed brain on glycolysis in muscle extract. *A*, no cytolysed brain; *B*, 0·2 ml. cytolysed brain susps.; *C*, 0·4 ml. cytolysed brain; *D*, 0·6 ml. cytolysed brain added to 2·5 ml. muscle extract.

showed very slight inhibitory action. Starting with small amounts of cytolysed brain it was observed that the inhibition of glycolysis was progressive. The rate of inhibition depended on the amount of cytolysed brain added, as shown in Fig. 1. In all experiments in which complete inhibition was observed the final

glycogen content of the reaction mixture was still high. This last fact indicates that the inhibiting factor is not amylase. The experiments are described in Tables III, IV and V. Brain was cytolysed by suspending the pulp in 5 vol. of distilled water. The extract from fresh muscles of rabbits or rats was prepared according to Meyerhof. In a large number of the experiments acetone-dried muscle extracts were used. The dry muscle powder was extracted with isotonic NaCl solution and was cleared by centrifuging. The glycolytic rate was determined chemically as well as by manometric methods. In several cases the glycogen content of the flasks was also determined at the end of the experiment. In all the experiments where lactic acid was determined chemically, 1 ml. of muscle extract was made up with $M/15$ phosphate buffer of pH 7·2, or with bicarbonate-Ringer, and with other additions to 5 ml. Glutathione and AP were added to each flask. For brevity's sake only one experiment from a larger number of uniform results is given.

Table III

	mg. lactic acid formed in 1 hr.	mg. glycogen found at start of exp.	μl. CO_2 evolved in 1 hr.	mg. glycogen found at end of exp.
Muscle extract:	(1) Glycogen			
Without brain	2·74	156	122	38
With 0·04 g. cytolysed brain	1·21	138	46	29
With 0·06 g. cytolysed brain	0·18	129	0	24
	(2) Hexosediphosphate			
Without brain	1·89	—	108	—
With 0·04 g. brain cytolysate	1·50	—	91	—
With 0·08 g. brain cytolysate	1·04	—	35	—
With 0·12 g. brain cytolysate	0·29	—	0	—
	(3) α-Glycerophosphate			
With α-glycerophosphate	1·63	—	68	—
With 0·08 g. cytolysed brain	0·81	—	31	—
Cytolysed brain	0·44	—	11	—
	(4) α-Glycerophosphate and pyruvic acid			
Muscle extract	2·28	—	132	—
With 0·08 g. brain cytolysate	1·25	—	63	—
With 0·12 g. brain cytolysate	0·64	—	26	—

The amounts of cytolysed brain stated in the table were added to each ml. of muscle extract.

As Table III shows, the inhibitor cannot be amylase as there is still a considerable amount of glycogen left when inhibition is complete, and, as lactic acid formation is inhibited from both hexosediphosphate and from α-glycerophosphate. Larger amounts of cytolysed brain are necessary to inhibit lactic acid formation from hexosediphosphate than from glycogen.

Inhibition of lactic acid formation from glucose in muscle extract in the presence of hexokinase

Cytolysed brain also inhibits lactic acid formation in muscle extracts from glucose in the presence of hexokinase. In these experiments acetone-dried muscle extract was used. The hexokinase was prepared according to Meyerhof [1927].

Table IV. *Inhibition of lactic acid formation from glucose in the presence of of hexokinase*

	mg. lactic acid formed in 1 hr.
Muscle extract, glucose, AP and hexokinase	7·42
As above, with the addition of 0·1 g. cytolysed brain	0·66

Inhibition of fermentation in yeast extract

Fermentation in yeast extracts is also strongly inhibited by cytolysed brain. In one experiment, for instance, 289 μl. CO_2 were evolved without the addition of cytolysed brain, and only 20 μl. in the presence of cytolysed brain.

Effect of cytolysed brain on glycolysis of intact brain cells and on the fermentation in living yeast

Glycolysis and fermentation of intact cells cannot be inhibited by cytolysed brain, hence the inhibitory substance cannot pass through cell membranes. An experiment of this type is described in Table V.

Table V

	μl. CO_2 evolved
Brain pulp in Ringer solution	123
As above, with 0·3 g. cytolysed brain	132
Living beer yeast	186
Living beer yeast with 0·3 g. cytolysed brain	194

Effect of coenzymes

By the addition of adenosinetriphosphate, of cozymase, of glutathione and of creatinephosphate it was attempted to reactivate muscle extracts which had been inactivated by cytolysed brain. Different amounts of the coenzymes were used. The maximum amount of cozymase added to 2·2 ml. fluid was 10 mg. of a pure product. In no case could any reactivation be observed. In these experiments, as in the former ones with brain cytolysate alone, an increased CO_2 output was observed after adding cozymase. Chemical analysis showed, however, no corresponding increase in lactic acid formation. For the sake of brevity these experiments are not described here.

The facts described in this section indicate a difference in behaviour between tumour cells and brain tissue. Cozymase and adenylic acid do not reactivate cytolysed brain although they did reactivate tumour extracts in the experiments of Boyland *et al.* [1937]. On the other hand brain cells do not form lactic acid from hexosediphosphate, while according to Boyland & Boyland tumour extracts do, after the addition of adenylic acid and cozymase.

Preparation of antiglycolytic extracts from cytolysed brain

Cell-free aqueous extracts of cytolysed brain possess very slight antiglycolytic activity. Repeated washing of cytolysed brain with water only slightly diminishes its inhibitory power. Acid extraction has no inhibitory effect. On the other hand, alkaline extracts show a considerable inhibiting activity. Experiments were made as follows: finely ground pulp of fresh brain cortex was suspended in four parts of distilled water or in 0·1 *N* acetic acid or in 0·6 % $NaHCO_3$. After 15 min. at room temperature, part of the suspension was pipetted off and the remaining part centrifuged. The clear supernatant extract was pipetted off and the residue filled up to its original volume. In a few cases this last procedure was repeated several times. To test the inhibitory effect of brain extracts, they were added to muscle extract containing glucose, hexokinase and AP. The experiment in Table VI was made manometrically. The total volume of fluid in the manometer vessels was 3·2 ml.

Table VI

	μl. CO_2 evolved in 1 hr.
Muscle extract, glucose, hexokinase, AP	118
With 0·2 ml. of cytolysed brain suspension (1 : 5)	0
With 0·5 ml. of water extract of cytolysed brain	109
With 1·5 ml. of water extract of cytolysed brain	85
With 1·5 ml. of acid extract of cytolysed brain	109
With 0·4 ml. of alkaline extract of cytolysed brain	35
With 0·6 ml. of alkaline extract of cytolysed brain	3

As Table VI shows, extracts possessing about a third of the inhibitory action of the original suspension may be obtained by extraction with 0·5 % $NaHCO_3$ solution.

The effect of heating and of drying

Heating of the cytolysed brain suspension or extract for 3 min. to 100° completely destroys its inhibiting power. On the other hand, in the heated suspension the presence of a substance which reactivates aged muscle extracts can be demonstrated. This activator can be best demonstrated on rat muscle extract which has been kept for 2 days previously in a refrigerator. One such experiment is shown in Table VII. It seems that this activator is not AP and not cozymase, as neither of these substances possesses the same activating effect as boiled brain extract. Precipitation and drying with acetone destroy a great part of the inhibiting effect of the extract. The inhibitory substance does not pass through collodion membranes.

Table VII. *Activating effect of heated brain extract*

	mg. lactic acid produced in 1 hr.
Aged rat muscle extract with AP and cozymase	39
Same as above with the addition of 0·4 ml. heated brain extract	81

The effect of p*H on inhibition*

The inhibitory effect of cytolysed brain on muscle glycolysis is greatest at *p*H 7·0–7·5. The experiment in Table VIII was made in *M*/15 phosphate buffer. The amount of cytolysed brain used was chosen so as to cause about 50–70 % inhibition.

Table VIII. *Effect of* p*H on inhibition*

	mg. lactic acid formed in 1 hr.
*p*H 6·5 without inhibitor	1·42
with cytolysed brain	0·83
*p*H 7·0 without inhibitor	3·35
with cytolysed brain	1·31
*p*H 7·5 without inhibitor	4·70
with cytolysed brain	2·24
*p*H 8·0 without inhibitor	5·70
with cytolysed brain	4·14

Discussion

The facts described in this paper prove that intact cell structure is necessary for brain glycolysis. By destruction of the cells a substance is liberated which inhibits glycolysis. No indication can be given of the nature of the inhibitory

substance although it is fairly well established that it is not amylase. It appears also that the inhibitor is different from the nucleosidase of tumour, described by Boyland & Boyland [1935]. While the nucleosidases reduce methylene blue in the presence of adenosinetriphosphate and of cozymase, no such reduction can be observed with cytolysed brain. The inhibitory action of cytolysed brain cannot be counteracted or the system reactivated by adenosinetriphosphate or by cozymase. On the other hand these substances reactivate tumour extract according to Boyland *et al.* [1937]. The glycolysis inhibitor of brain cannot be antiglyoxalase, since cytolysed brain possesses a considerable glyoxalase activity. Several facts, however, indicate the enzymic nature of the brain inhibitor. It is destroyed when heated for 2–4 min. to 100°. It does not pass through a collodion membrane. It loses part of its activity when dried and it has a *p*H optimum. The inhibition caused by cytolysed brain in muscle extract is a gradual one. The time at which the inhibition is complete depends on the amount of cytolysed brain present. This last fact indicates an enzymic destruction of a component of the glycolytic system.

As the inhibitory substance is indiffusible through collodion membranes it is evidently unable to penetrate cell membranes. In fact, cytolysed brain inhibits glucose fermentation in yeast extract, but it is unable to inhibit the fermentation of living yeast, and it does not inhibit glycolysis in a suspension of intact brain cells. These facts make it clear that extraction of the glycolytic system from brain causes at the same time liberation of an inhibitory substance which destroys the glycolytic activity.

Summary

1. Brain tissue loses its glycolytic power if the cell structure is destroyed by grinding with sand, or freezing and thawing or by suspending it in water. The cytolysed brain cannot convert any of the zymophosphates into lactic acid.

2. The cytolysed brain tissue does not oxidize glucose. Lactate oxidation is also greatly reduced. Cytolysis has no effect upon succinate oxidation.

3. The glycolytic activity and the respiration of the brain which are lost by cytolysis cannot be restored by cozymase or by adenosinetriphosphate or glutathione.

4. The addition of small amounts of cytolysed brain to a glycolysing muscle extract inhibits the glycolysis of the latter. Lactic acid production, whether from glycogen, from hexosediphosphate, from α-glycerophosphate and pyruvic acid, or from glucose with hexokinase, is inhibited by cytolysed brain.

5. Cytolysed brain inhibits the glucose fermentation of yeast extract.

6. Cytolysed brain does not inhibit the lactic acid formation by intact brain cells or the fermentation of living yeast.

7. Neither cozymase, adenosinetriphosphate, glutathione nor creatine-phosphate reactivates muscle extracts inhibited by cytolysed brain.

8. In boiled extract of brain the presence of a substance can be demonstrated which restores the glycolytic power of aged muscle extracts. The activator is neither cozymase nor adenosinetriphosphate.

9. With hypotonic $NaHCO_3$ a cell-free extract of the glycolysis inhibitor can be prepared from cytolysed brain.

10. The inhibitor is destroyed by heat, and is partly destroyed by dehydration with acetone. It does not diffuse through collodion membranes.

11. The optimum *p*H for inhibition is 7·0–7·5.

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CVII. METABOLISM OF SULPHUR

VII. A QUANTITATIVE STUDY OF THE REPLACEABILITY OF *l*-CYSTINE BY VARIOUS SULPHUR-CONTAINING AMINO-ACIDS IN THE DIET OF THE ALBINO RAT[1]

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(*Received 11 March 1939*)

In experiments reported previously on the replaceability of *l*-cystine in the diet of albino rats with some partially oxidized derivatives [Bennett, 1937], the compounds fed were *l*-cystine disulphoxide, *l*-cysteinesulphinic acid and S-(guanylthio)-cysteine.2HCl, which gives rise to *l*-cysteinesulphenic acid upon hydrolysis. *l*-Cystine disulphoxide proved capable of replacing *l*-cystine in the diet; *l*-cysteinesulphinic acid produced no growth; *l*-cysteinesulphenic acid resulted in a slight but definite increase in growth. These compounds were fed in sulphur equivalents that were in excess of the minimum amount necessary for maximum growth; therefore the results could not be interpreted quantitatively. In the present series of experiments, cysteine, cystine disulphoxide and methionine were fed, the basal diets being supplemented with smaller graded amounts of these amino-acids, in an attempt to demonstrate quantitative relationships in their ability to promote growth.

Preparation of compounds

The compounds employed were: *l*-cystine (Merck); *l*-cystine disulphoxide (Merck); *l*-cysteine prepared from cysteine hydrochloride [Toennies & Bennett, 1936]; *dl*-methionine (Eastman), 98·0% purity according to sulphur determination;[2] *l*-methionine, isolated by Dr Toennies from egg albumin, 99·9% purity, Exp. 2; 97·0% purity, Exp. 4, according to sulphur determinations.[2] Arachin isolated from peanut meal[3] by the method of Johns & Jones [1916], 0·77% methionine and 0·92% cystine according to method of Kassel & Brand [1938].[2]

Experimental

Albino rats, Wistar strain, 25 days old, were used as experimental animals. In Exps. 1–3 they were kept on Dyer & du Vigneaud's [1936] cystine-deficient basal diet containing 6% casein and 16% milk vitamin concentrate fed *ad libitum*. In Exp. 4 the basal diet was methionine-deficient; 15% arachin was used instead of casein as the basal protein and the dextrin was reduced to 24%; each rat received 100 mg. Harris vitamin B complex daily. Sufficient dry

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[2] I am indebted to Mr Thomas P. Callan for these determinations.

[3] I wish to thank the Planters Peanut Co., Norfolk, Va., for the gift of the peanut meal.

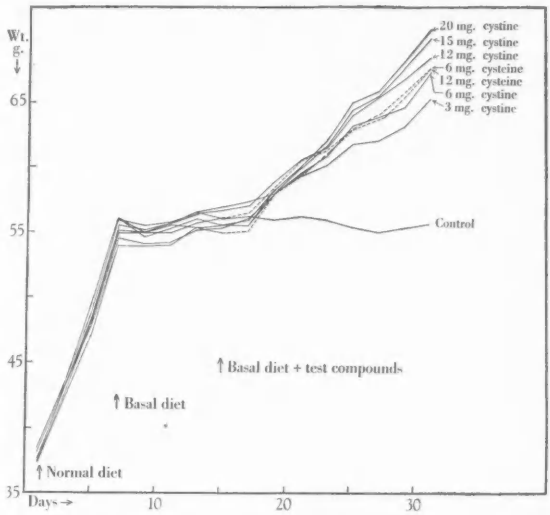

Fig. 1. Determination of the amounts of *l*-cystine and *l*-cysteine just adequate for maximum growth. Du Vigneaud's basal diet was used.

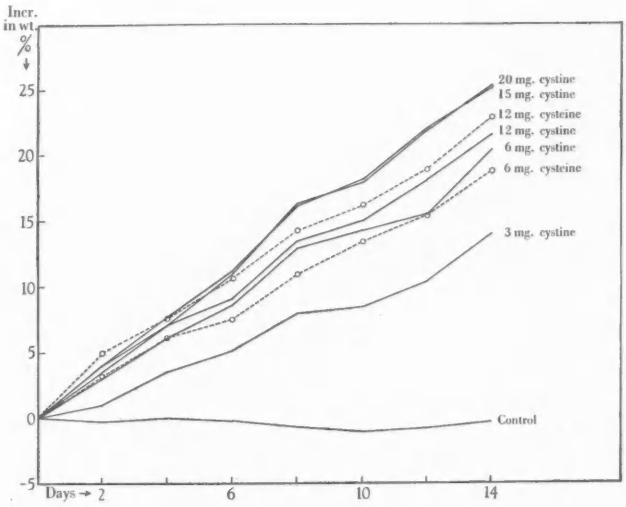

Fig. 1*a*. Fig. 1 recalculated, expressing the increase in weight in percentage of the weight at the beginning of the special diets.

mixture was made at the beginning of each experiment to last through the entire period. From this, fresh basal diets were prepared every 3 days. All food was kept in the refrigerator. The special compounds were fed individually in small pieces of butter, the control animals receiving the same amount of butter. On account of the extreme lability of the sulphur compounds, especially the cysteine, great care was taken to prevent decomposition. These butter mixtures were made once a week, keeping all instruments on ice during the mixing, and were kept in individual covered glass dishes in the refrigerator at 0°, the more labile at −10°. The animals were maintained on a normal diet for 8 days, at the end of which the basal diet was supplemented with the special compounds for a period of 2 weeks. The rats were weighed every other day and the average weight of the group plotted.

Exp. 1. *To determine the amount of cystine just adequate for maximum growth.* In the first experiment, 96 rats from 11 litters, 25 days old, were divided into 8 comparable groups of 12 animals each, 6 males and 6 females. After 8 days on the cystine-deficient diet, cystine was fed in addition to the basal diet in 20, 15, 12, 6 and 3 mg. portions daily to five groups respectively. Two additional groups received 12 and 6 mg. of *l*-cysteine; one group was continued unsupplemented (Figs. 1, 1*a*). In Fig. 1*a*, the increase in weight is expressed in percentage of the weight at the beginning of the special diets. This allows the curves to start from a single point and brings out the true grouping which is slightly changed from that of the other graph. This experiment showed that 15 mg. of cystine added daily in excess of the basal diet produced maximum growth. It also brought out that the upper portion of the range was less suited to quantitative measurements than the lower in which slight differences in intake might produce greater spread of the curves. Therefore the scale of reference for the succeeding experiments was reduced to 12, 6, 3 and 1·5 mg. of cystine. The maximum amount of cystine added to these figures, due to the daily consumption of 5–10 g. of du Vigneaud's basal diet, would not exceed 1·8 mg. of cystine. These amounts of cystine are below the level of those used in most growth experiments reported in the literature. For instance, du Vigneaud, in his studies, fed the animals 20 mg. of cystine, or its sulphur equivalent, daily in addition to the cystine in the basal diet [Loring *et al.* 1933; Dyer & du Vigneaud, 1935; 1936]. A computation of the amounts of cystine ingested daily by the rats used by Womack *et al.* [1937] in their study of the growth-promoting potencies of cystine and methionine, show (their Tables II and III) that on an average approximately 12–16 mg. of cystine were consumed daily by each rat.

Exp. 2. *To determine the quantitative metabolic relationships of* l-*cystine,* l-*cysteine,* l-*cystine disulphoxide, and* l-*methionine, as expressed by growth curves.* In the second experiment, 88 rats from 12 litters, 25 days old, were divided into 11 comparable groups of 8 animals each, 4 males and 4 females. After 8 days on the cystine-deficient diet, the various groups, with the exception of the control group, were supplemented daily. Four groups received 12, 6, 3 and 1·5 mg. of cystine respectively. The other six groups were fed daily 3·0 and 12·1 mg. of *l*-cysteine; 3·7 and 14·9 mg. of *l*-methionine; and 3·4 and 13·6 mg. of cystine disulphoxide, respectively; amounts containing the sulphur equivalents of 3 and 12 mg. of cystine. Fig. 2 gives the customary type of growth curve; Fig. 2*a* shows the increase in weight expressed in percentage of the weight at the beginning of the special diets. As shown, all the curves representing 12 mg. equivalents (letter *D*) fall in a group as do those corresponding to 3 mg. (letter *B*). In both groups disulphoxide curves fall lower as the experiment progresses.

Exp. 3. *Repetition of Exp. 2 with* dl-*methionine in place of the* l-*form.* In the third experiment, 110 rats from 13 litters, 25 days old, were divided into 11

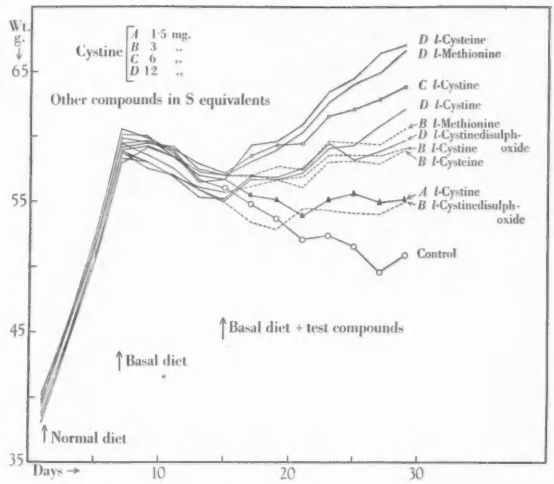

Fig. 2. Growth curves expressing the quantitative metabolic relationships of *l*-cystine, *l*-cysteine *l*-cystine disulphoxide and *l*-methionine. Du Vigneaud's basal diet was used.

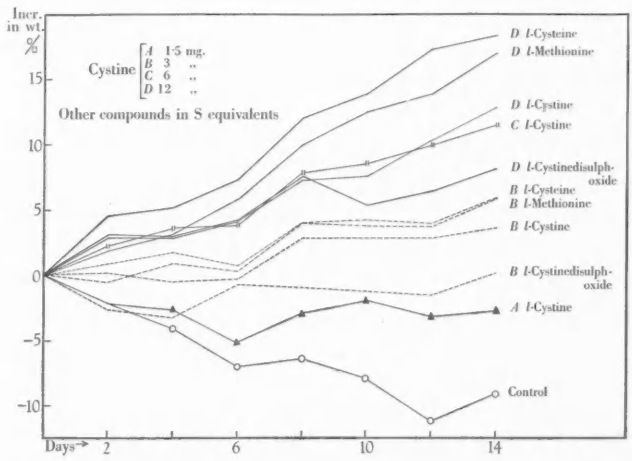

Fig. 2*a*. Fig. 2 recalculated, expressing the increase in weight in percentage of the weight at the beginning of the special diets.

comparable groups of 10 animals each, 5 males and 5 females. The various groups were treated as in Exp. 2; the compounds and amounts fed were the same, with the exception of methionine. The results are depicted in Fig. 3. The curves

representing the 12 mg. equivalents fall in a group as before and about the same growth is attained in all cases, indicating that with this intake all the compounds are present in sufficiently large amounts to insure maximum growth. In the group of curves representing the 3 mg. intake, however, a spreading occurs. The cystine disulphoxide curve, as before, is the lowest, dropping below that for 1·5 mg. cystine, nearer the probable location of a 1 mg. cystine curve. The 3 mg. *dl*-methionine curve falls slightly above the 1·5 mg. cystine curve.

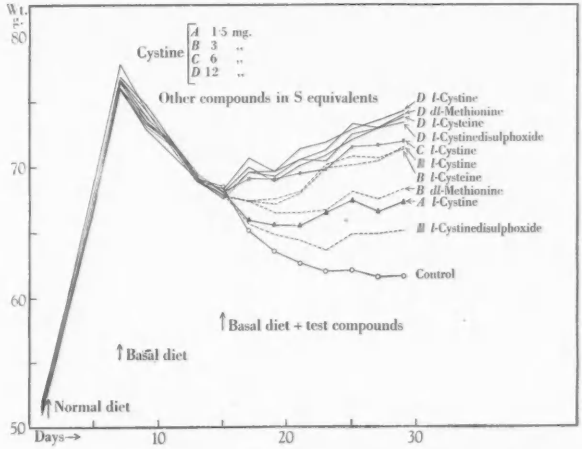

Fig. 3. Growth curves expressing the quantitative metabolic relationships of *l*-cystine, *l*-cysteine, *l*-cystine disulphoxide and *dl*-methionine. Du Vigneaud's basal diet was used.

Exp. 4. *To determine whether* dl-*methionine is as readily utilized for growth as the* l-*form*. A comparison of the growth curves of rats receiving the smaller amount of *l*-methionine in Exp. 2, and *dl*-methionine in Exp. 3, suggests that the *dl*-methionine may not be exactly the equivalent of the *l*-form. Since these curves are from separate experiments, it was decided to test this more adequately by feeding the *l*- and *dl*-forms of methionine in one experiment (4). The casein used in the basal diet provided 1·9 mg. of methionine for each g. of basal food. In an attempt to reduce this basal intake, arachin was substituted for casein since it is said to contain 0·54 % methionine [Baernstein, 1932] amounting to about 0·8 mg. of methionine per g. of basal food. The arachin isolated for the present experiment, however, showed 0·77 % methionine by Kassel & Brand's [1938] modification of Baernstein's [1932] method or 1·2 mg. of methionine per g. of basal food. Twenty-five male rats, 23 days old, from 5 litters were divided into 5 groups, one rat from each litter in each group. After 8 days on the methionine-deficient diet, the various groups, with the exception of the control, were supplemented daily. Groups B and C received 6 and 12 mg. of *l*-methionine, respectively, and D and E received the same amounts of *dl*-methionine daily. The approximate amount of the basal diet consumed per rat per day was determined by weighing daily the basal food given each group and the residual food, and dividing by the number of animals in the group (Table I). This experiment was conducted on a methionine-deficient diet, with arachin as the basal protein. Fig. 4 gives the curves obtained.

Table I. *Daily basal food consumption per rat in g. Averaged over 2-day period. Exp. 4*

	Unsupplemented				
Days	Group A	Group B	Group C	Group D	Group E
1–2	4·1	4·5	5·2	4·7	4·6
2–4	4·9	5·4	5·7	5·4	5·4
4–6	4·7	5·2	4·9	5·5	5·0
6–8	4·4	5·9	5·9	5·3	5·2

		Daily supplement			
	Unsupplemented Control	*l*-Methionine, mg.		*dl*-Methionine, mg.	
		6	12	6	12
8–10	4·5	6·4	6·2	5·4	5·8
10–12	4·7	6·8	5·4	5·2	6·2
12–14	5·0	6·2	6·3	5·5	6·6
14–16	4·8	5·8	5·7	6·0	6·6
16–18	4·7	6·2	6·2	6·1	7·3
18–20	4·4	6·3	6·3	5·9	7·4
20–22	4·8	6·6	6·8	6·4	7·8

Table II. *Relation of growth to food consumption. Averaged per rat over a 14-day period of feeding. Exp. 4*

Group	Daily supplement	Increase in weight g.	Food consumption g.	Gain per g. of food consumed g.
B	6 mg. *l*-methionine	11·2	88·6	0·126
C	12 mg. *l*-methionine	13·1	85·8	0·152
D	6 mg. *dl*-methionine	8·1	81·0	0·100
E	12 mg. *dl*-methionine	12·4	95·4	0·129

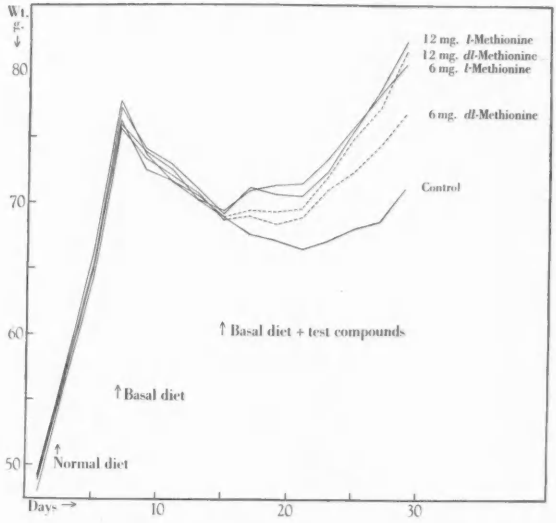

Fig. 4. A comparison of the growth-promoting properties of *l*-methionine and *dl*-methionine. The casein of du Vigneaud's basal diet was replaced by arachin.

Discussion

There has been some question as to whether the cystine-cysteine relationship in metabolism is solely an oxidation-reduction relationship or whether hydrolytic cleavage of cystine also plays a part. In the former case, one molecule of cystine should be the equivalent of two molecules of cysteine; in the latter case, a one to two relationship would not hold. Shinohara & Kilpatrick [1934], working in acid solution without addition of heavy metals, reported that through hydrolysis and subsequent dismutation three molecules of cystine gave rise to five of cysteine and one of cysteic acid. This was in agreement with earlier work of Vickery & Leavenworth [1930] who worked with heavy metals in solution, whereby cysteine was removed from the mixture as rapidly as it was formed. Lavine [1937], also with heavy metals present, found that two molecules of cystine gave rise to three molecules of cysteine and one of sulphinic acid. In Exps. 2 and 3 the clear-cut separation of the curves into two groups representing those rats which received the equivalents of 12 mg. cystine and those which received the equivalents of 3 mg. indicates (Figs. 2, 2*a*, 3) that, under these conditions, two molecules of cysteine are fully the equivalent of one of cystine.

Whether oxidation of disulphoxide sulphur ever occurs *in vivo* is uncertain. Evidence from the present experiments shows that the formation of cystine from its disulphoxide can occur, although one molecule of cystine disulphoxide did not give rise to the same amount of growth as did one molecule of cystine. Possibly a series of reactions takes place in the body similar to that found in test-tube experiments by Lavine [1936], whereby three molecules of cystine disulphoxide, through hydrolysis and dismutation, give rise to four molecules of cysteinesulphinic acid and one molecule of cystine.

The results of Exp. 4 substantiate those of Exps. 2 and 3; the *dl*-methionine when fed in submaximal amounts did not produce quite as much growth as the *l*-form. Since both the *l*- and the *dl*-forms are oxidized to sulphate [Stekol, 1935], it might seem reasonable to suppose that they have equal growth-promoting properties. However, it is known that the readiness with which a compound is oxidized and its availability for growth do not run parallel in some compounds. For instance, du Vigneaud *et al.* [1934] found that *S*-methylcysteine is readily oxidized, but that feeding this compound does not bring about growth of rats on a cystine-deficient diet.

Considering the relation of growth to food consumption (Exp. 4, Table II) the gain in weight for each g. of food consumed is practically the same with an intake of 6 mg. *l*-methionine (group B) and 12 mg. *dl*-methionine (group E), 0·126 and 0·129 g. respectively. The lowest relative gain was attained with the 6 mg. *dl*-methionine supplement, or 0·10 g. for each g. of food ingested. An intake of 12 mg. *l*-methionine definitely increased the utilization of food for growth; these animals show 0·15 g. increase in weight for each g. of food consumed. Kik [1938] similarly reported a better utilization of food when a casein ration was supplemented by methionine than when supplemented by cystine. Animals on the 12 mg. *dl*-methionine, after several days, grew at approximately the same rate as those on 12 mg. *l*-methionine; it is attained, however, only by increasing intake, 85·8 g. of food for those on 12 mg. *l*-methionine compared with 95·4 g. for those receiving 12 mg. *dl*-methionine. Growth curves reported by Jackson & Block [1938] demonstrate this same tendency. Their rats receiving the *d*-methionine, increase their daily food consumption to a greater extent than those receiving the *l*-methionine, while gaining approximately the same number

of g. in weight. This tendency can be seen by a rough estimation of g. gained and daily food consumption given in Fig. 1 of their paper.

From Exp. 4 some idea of the absolute amounts of a mixture of cystine and methionine which will produce normal growth in albino rats may be obtained. Calculating the amounts of methionine and cystine consumed in the basal diet in addition to those fed as supplement, Group C had a daily intake of 19·3 mg. *l*-methionine and 8·5 mg. *l*-cystine; group E, 12 mg. *dl*-methionine, 8·2 mg. *l*-methionine, and 9·5 mg. *l*-cystine; group B, 13·6 mg. *l*-methionine and 8·8 mg. *l*-cystine. These three groups undergo practically the same increase in weight during the experimental period. The last group D, which received a daily intake of 6 mg. *dl*-methionine, 7·0 mg. *l*-methionine and 8·1 mg. *l*-cystine, grew somewhat less; therefore, in this case, we conclude that methionine and cystine were present in suboptimal amounts.

Summary

Under the conditions of the experiments reported, the following quantitative metabolic relationships are indicated as expressed by growth curves:

One molecule of *l*-cystine is available from three molecules of *l*-cystine disulphoxide.

One molecule of *l*-cystine is equivalent to two molecules of *l*-cysteine.

One molecule of *l*-cystine is equivalent to two molecules of *l*-methionine.

dl-Methionine is somewhat less readily utilized for growth than is the *l*-form.

l-Methionine definitely increases the utilization of food for growth.

The absolute amounts of methionine and cystine, which permit normal growth in young albino rats on an otherwise adequate diet under the conditions of the last experiment, are estimated.

The author wishes to thank Dr Medes for her advice and criticism.

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CVIII. STUDIES ON PROTEINASES OF SOME ANAEROBIC AND AEROBIC MICRO-ORGANISMS

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Clostridium histolyticum secretes a proteinase, with a *p*H optimum of 7, which can be activated markedly by —SH compounds; and this activation can be increased by the addition of traces of heavy metal [Weil & Kocholaty, 1937; Kocholaty, Weil & Smith, 1938]. The results of the present study, an investigation of the proteinases of several other Clostridia, indicate that the proteinases of some other members of this genus have similar *p*H optima and activation characteristics.

Cell-free filtrates of *Pseudomonas fluorescens*, *Serratia marcescens*, *Bacillus mycoides* and *Staphylococcus citreus* contain proteinases which, although differing in their partial activation behaviour (with cysteine alone and Fe^{++} alone), as a whole resemble the proteinases of the members of the *Clostridium* group.

Experimental

The bacterial proteinase solutions were prepared in the same manner as those previously reported [Weil & Kocholaty, 1937] by inoculating the bacteria into culture media and filtering through a Seitz filter after 18–24 hr. anaerobic incubation at 37°. For *Cl. histolyticum* and *Cl. Welchii*, horse meat broth was used; for *Cl. sporogenes*, a 3 % solution of Difco neopeptone; for *Cl. putrificum* and *Cl. botulinum*, beef heart infusion broth. The *p*H of the culture media was adjusted to about 7·4. The organisms used were obtained from the American Type Culture Collection. The strain of *Cl. histolyticum* was in the rough phase [Hoogerheide, 1937].

One ml. of *Cl. histolyticum*, 2 ml. of *Cl. Welchii* and 3 ml. of *Cl. sporogenes* or *Cl. botulinum* filtrates, respectively, were taken for each determination. To this amount of bacteria-free filtrate, either 10 mg. of cysteine-HCl (neutralized), 0·6 ml. of 0·1 *N* $FeSO_4$, or both, were added. After the addition of 3 ml. of citrate-phosphate buffer at the proper *p*H, 3 ml. of a 3 % gelatin solution of the same *p*H were pipetted into the reaction mixture. The total volume was made up to 10 ml. After an incubation of 22 hr. in a hydrogen atmosphere at 37°, the increase of amino groups was measured by the Van Slyke method, and the results were expressed in ml. of 0·05 *N* KOH. All the filtrates of the Clostridia were practically free of peptidases, and the activities measured in this way were attributable to the proteinase action alone.

From Table I it can be seen that the proteinases of the Clostridia resemble very much, with respect to *p*H optima and activations, the proteinase of *Cl. histolyticum*, already described [Weil & Kocholaty, 1937].

Maschmann [1938, 1] stated that the cell-free filtrate of *Cl. Welchii* without activation could hydrolyse only gelatin, although, when activated by —SH compounds, clupein was hydrolysed. The other protein substrates that he

Table I. *Activation and pH optima of the proteinases of some Clostridia measured on gelatin*

	*p*H...	4	5	6	7	8
	Activator	Activity in ml. of 0·05 *N* KOH				
Cl. histolyticum	None	0·0	0·56	3·52	4·32	3·62
	Cysteine	0·0	1·20	4·84	5·62	4·98
	Fe^{++}	0·0	0·52	3·20	4·00	3·08
	Cysteine-Fe^{++}	0·10	1·74	5·82	7·16	5·64
C. Welchii	None	0·04	—	0·94	1·20	1·00
	Cysteine	0·20	—	1·96	2·24	2·04
	Fe^{++}	0·24	—	3·22	3·70	3·12
	Cysteine-Fe^{++}	0·24	—	3·00	3·28	2·74
Cl. sporogenes	None	0·11	—	2·10	2·44	2·01
	Cysteine	0·14	—	3·06	3·58	3·12
	Fe^{++}	0·22	—	4·01	4·22	3·82
	Cysteine-Fe^{++}	0·30	—	5·81	6·46	5·76
Cl. putrificum	None	0·00	—	1·37	1·65	1·01
	Cysteine	0·09	—	1·72	2·04	1·29
	Fe^{++}	0·32	—	2·41	2·81	1·68
	Cysteine-Fe^{++}	0·22	—	2·16	2·36	1·64
Cl. botulinum	None	0·08	—	0·49	0·77	0·56
	Cysteine	0·17	—	0·67	0·97	0·76
	Fe^{++}	0·30	—	0·83	1·18	0·98
	Cysteine-Fe^{++}	0·24	—	0·81	1·16	0·99

investigated (ovalbumin, casein, Witte peptone) were but slightly hydrolysed. The proteinase responsible for gelatin hydrolysis was found to be secreted into the medium in the early stages of bacterial growth. However, the proteinase which induces the hydrolysis of clupein was described as appearing only late in incubation, presumably after autolysis of the bacteria had occurred. From this, Maschmann assumed that this latter proteinase was an intracellular one. Similar conclusions were reached [Maschmann, 1938, 2] in connexion with the proteinases of *Cl. histolyticum* and *Cl. botulinum*.

Because of this description of an intracellular proteinase which promotes the hydrolysis of clupein, and of our previous negative results in regard to this point [Weil & Kocholaty, 1937; Kocholaty, Weil & Smith, 1938], careful attention has been given, in the present study, to factors affecting autolysis.

A cell-free filtrate of *Cl. Welchii*, obtained from a 16 hr. culture, was used as a source of proteinase. At this time, 16 hr. after inoculation, practically all of the bacteria were living and dividing, and consequently, any enzyme present in the culture filtrate may be presumed to have been produced extracellularly. Two ml. of the *Cl. Welchii* filtrate were used for each enzyme activity determination. The activations were carried out as described above. After the addition of 3 ml. of citrate-phosphate buffer at *p*H 7·0, 3 ml. of a 5 % solution of gelatin, clupein sulphate, Witte peptone or casein at *p*H 7·0 were added. After 20 hr. of anaerobic incubation at 37°, the increase in amino groups was measured by the Van Slyke method. In the case of casein, alcoholic titration was used and the buffer was omitted, because of its disturbing effect on the titration. The *p*H, however, was adjusted in this special case.

As Table II shows, the proteinase of *Cl. Welchii* readily hydrolysed all the investigated proteins, and in every case a marked activation was obtained with cysteine-Fe^{++}. The similar behaviour of the enzyme-activator system toward the investigated protein substrates did not indicate a number of proteinase actions. This enzymic picture resembles very much that obtained in our previous work [Weil & Kocholaty, 1937] on the proteinase of *Cl. histolyticum*.

Table II. *The decomposition of various protein substrates by* Cl. Welchii *proteinase*

	Activator			
	None	Cysteine	Fe^{++}	Cysteine-Fe^{++}
Substrate	Activity in ml. of 0·05 *N* KOH			
Gelatin	0·84	1·91	1·01	1·91
Clupein	0·57	1·65	0·77	2·19
Witte peptone	0·60	0·00	0·10	1·40
Casein	0·56	1·28	0·76	1·32

A recent paper by Berger *et al.* [1938] also indicates a possible importance of reducing substances for the peptidase system of anaerobic bacteria. A similar observation was reported by Maschmann [1938, 3], and he made the suggestion that the activation observed by us on the proteinase of *Cl. histolyticum* was due to the peptidase system. However, such an explanation does not hold for the proteinases of Clostridia. Bacteria-free filtrates of the *Clostridium* group after 1 day's cultivation were found to contain practically no peptidase, whether tested for in the presence or absence of the activator system. The peptidases, as has been shown [Kocholaty, Smith & Weil, 1938], are intracellular, and appear in the bacterial filtrate only after the autolysis of the bacteria.

Further evidence that the activation is due to the proteinase action was given by the liquefaction of gelatin by the proteinase of *Cl. histolyticum*. The

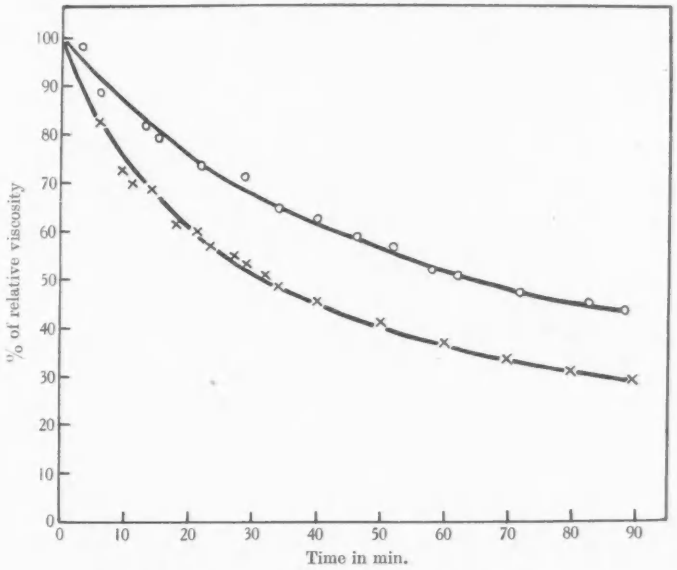

Fig. 1. o—o Without activator. × — × With activator.

hydrolysis of gelatin by *Cl. histolyticum* proteinase, measured by the viscosimetric method, was markedly activated by cysteine-Fe^{++}. This method of proteinase determination measures the very first step of protein breakdown, one

which is indeterminable by any chemical method. The activation of this first step of hydrolysis cannot, therefore, be explained by a peptidase action. One ml. of *Cl. histolyticum* filtrate, diluted 1:80, and 3 ml. of 3 % gelatin solution at *p*H 7·0 were used, the total volume being 4 ml. The activation was carried out as usual. The drop in the viscosity was measured at 37° and is expressed in percentage of the original viscosity (Fig. 1).

Characteristics of the proteinases of some aerobic bacteria

For the preparation of proteinase of *B. mycoides*, a pure culture was inoculated into a medium containing 0·3 % beef extract, and 0·5 % yeast extract. After an aerobic incubation of 5 days at 37°, the culture was filtered. Bacteria-free proteinase solutions of *Staph. citreus*, *Ps. fluorescens*, *S. marcescens* and *Proteus vulgaris* were obtained through inoculation of the bacteria into heart-infusion broth. After 48 hr. aerobic incubation at 37°, the cultures were filtered. For each enzyme determination 3 ml. of the filtrate were used, and the estimations were carried out in the same way as those described for the proteinases of members of the *Clostridium* group.

Table III, which represents the activations and *p*H-optima of proteinases derived from *B. mycoides*, *Staph. citreus*, *Ps. fluorescens*, and *S. marcescens*, presents a uniform picture. The addition of cysteine, which activated the proteinases of the *Clostridium* group, produced in these cases a partial inhibition, while Fe^{++} had an activating effect. Cysteine-Fe^{++} gave the maximal activation. The mechanism of these activations, however, requires further investigation. The *p*H-optima of the investigated proteinases were at neutrality, which is in agreement with the results of Gorbach & Pirch [1936] and Maschmann [1937], who worked on the proteinase of *Ps. fluorescens*.

Table III. *Activation and pH-optimum of the proteinases of some aerobic bacteria, measured on gelatin*

	*p*H...	4	6	7	8
	Activator	Activity in ml. of 0·05 *N* KOH			
B. mycoides	None	0·00	0·24	0·48	0·36
	Cysteine	0·00	0·12	0·28	0·14
	Fe^{++}	0·00	0·40	0·65	0·49
	Cysteine-Fe^{++}	0·00	0·48	0·75	0·54
Staph. citreus	None	0·00	0·78	1·12	0·64
	Cysteine	0·00	0·64	1·06	0·54
	Fe^{++}	0·20	2·32	2·60	2·24
	Cysteine-Fe^{++}	0·28	1·61	3·40	2·46
Ps. fluorescens	None	0·00	0·40	0·68	0·46
	Cysteine	0·00	0·16	0·37	0·25
	Fe^{++}	0·11	0·80	1·21	0·78
	Cysteine-Fe^{++}	0·10	0·88	1·23	0·86
S. marcescens	None	0·00	0·91	1·61	1·36
	Cysteine	0·00	0·64	1·16	0·48
	Fe^{++}	0·00	1·61	2·23	1·82
	Cysteine-Fe^{++}	0·08	1·69	2·52	2·08

The results given in Table IV, on the proteinase of *Pr. vulgaris*, resemble very much those obtained with the proteinases of the Clostridia. The difference between the activation behaviour of the proteinase of *Pr. vulgaris* and the proteinases of the aerobic organisms may be due to the fact that *Pr. vulgaris* is a facultative micro-organism. It is, however, quite possible that, by cultivating a facultative micro-organism for many generations aerobically, a proteinase may

Table IV. *Activation and pH-optimum of the proteinase of* Pr. vulgaris

	*p*H... 4	6	7	8
Activator		Activity of ml. of 0·05*N* KOH		
None	0·00	0·41	0·75	0·48
Cysteine	0·00	0·40	0·86	0·49
Fe^{++}	0·00	0·37	0·68	0·40
Cysteine-Fe^{++}	0·00	0·99	1·56	1·13

be obtained which would have the characteristics of those obtained from the aerobic group of organisms. The possibility of such variability was demonstrated previously [Kocholaty & Weil, 1938].

Summary

Anaerobic organisms, including *Cl. histolyticum*, *Cl. sporogenes*, *Cl. Welchii*, *Cl. putrificum* and *Cl. botulinum*, secreted proteinases which were activated by cysteine. The combination of cysteine with Fe^{++} gave the maximum activation.

The exocellular proteinase of *Cl. Welchii* hydrolysed clupein, gelatin, casein and Witte peptone.

Proteinases of certain aerobic organisms, *B. mycoides*, *Staph. citreus*, *Ps. fluorescens* and *S. marcescens*, were partially inhibited by cysteine. However, marked activations were effected by cysteine in combination with Fe^{++}.

The proteinase of the facultative anaerobe, *Pr. vulgaris*, resembled the proteinases of the Clostridia in activation behaviour.

Optimal action at neutrality was observed in all the investigated bacterial proteinases.

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CIX. THE COMPOSITION OF THE CROP MILK OF PIGEONS

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Pigeon squabs are hatched in the undeveloped state but grow very rapidly during the first few days after hatching. During this period they are fed on a special secretion formed in the crop of the parent birds. This secretion, commonly called "pigeon's milk", is a white, slimy, caseous material formed by the desquamation of epithelial cells and may be regarded as an example of a sacrifice of cell structure in the parent for the nutrition of the young. The endocrine control of its formation has been investigated by Riddle *et al.* [1932]. Its general composition and nutritive properties have been studied by Reed *et al.* [1932] and by Dabrovska [1932]. The former workers found the material to contain dry matter 35·7%, with protein 52·7, fat 35·6 and ash 4·5% of the dry matter; Dabrovska found dry matter 23·3%, and protein 57·4, fat 34·2 and ash 6·5% of the dry matter. The material did not contain carbohydrates.

In the present work, the compositions of four samples, pooled from 115 birds (three from 30 birds each, one from 25), have been investigated in detail. The birds had been used for prolactin assays, in which activity was measured by the wet weight of the crop glands. The gland contents were separated and made available for analysis as soon as possible after killing the birds.

Treatment of samples and methods of analysis

The samples were examined for small pieces of broken grain which were removed by hand-picking and the wet samples were weighed. The samples were dried in dishes with intermittent stirring, first at 60° and finally at 100°, and the weight of dry matter was determined. The powdered material was exhaustively extracted in a Soxhlet extraction apparatus for 18 hr. with light petroleum (b.p. below 40°) and the solvent-free fat determined and used for analysis.

The residue was further extracted with a boiling mixture of 3 parts ethyl alcohol and 1 part benzene for 12 hr. and the solvent-free extract was weighed and used for analysis.

The dried residue was used for the proximate and detailed analysis of organic constituents and ash. No further purification of the residue was carried out before acid hydrolysis of the protein to determine the Hausmann nitrogen distribution, since the material contained 15% of nitrogen on a moisture and ash-free basis. The values of the humin nitrogen in all cases were satisfactorily low.

Ash constituents. The phosphorus and metallic radicles were determined on the ash by the usual standard methods, but chloride was determined on the original material by the open-Carius method, all results being calculated on the basis of the dry matter of the secretion. The compositions of the four samples are given in Table I.

Protein. The protein-rich, fat-free residues were hydrolysed (1 g. portions) with 20% HCl in an autoclave at 130° and the distribution of nitrogen in three groups was determined. The results are given in Table II.

Table I. *Proximate composition and detailed analysis of crop milk of pigeons*

Sample no.	I	II	III	IV	Lactose-free dried milk
No. of birds	30	25	30	30	—
Yield of wet material g.	37·7	10·6	29·4	43·4	—
Yield of dry material g.	10·54	5·61	8·49	12·35	—
Dry matter per bird g.	0·35	0·22	0·28	0·41	—
Dry matter %	27·9	53·1*	29·1	28·5	—
% of dry matter:					
Total lipoids	33·4	32·7	32·6	36·3	46·4
Ether-sol. lipoids	28·4	27·7	27·7	31·1	—
EtOH-C_6H_6-sol. lipoids	5·0	5·0	4·9	5·2	—
Nitrogen	9·38	9·47	9·54	9·10	—
Protein (N × 6·25)	58·6	59·2	59·6	56·9	42·9
Ash	4·81	4·45	4·41	4·76	10·7
Calcium	0·261	0·313	0·276	0·362	1·79
Potassium	0·976	0·957	1·022	1·028	2·00
Sodium	0·311	0·340	0·306	0·294	1·14
Phosphorus	0·987	1·039	1·009	1·037	1·43
Chloride	0·055	0·066	0·052	0·059	1·43
Starch	3·23	5·21	3·59	3·57	—
True protein as % of total protein	93·4	95·7	92·7	94·9	94·0
Total accounted for	99·0	101·6	100·2	101·5	—
Ca/P (atom. equiv.)	3·8	3·3	3·7	2·9	1·0

* Sample was partly dried before analysis.

Table II. *Analysis of protein of pigeon crop milk. Nitrogen distribution*

Sample no.	I	II	III	IV	
Protein in dry matter (N × 6·25) %	58·6	59·2	59·6	56·9	—
Nitrogen in ash- and fat-free dry matter %	14·78	14·74	15·15	15·33	—
% of total N:					Chicken muscle
Amide-N	9·2	7·7	7·8	6·6	7·5
Diamino-N	31·2	29·3	33·6	32·5	30·0
Monoamino-N	55·9	59·0	55·7	56·8	59·8
Humin-N	3·7	4·0	2·9	4·1	2·7

Table III. *Analysis of lipoid extracts of pigeon crop milk*

Sample no.	I	II	III	IV
Total fat in wet matter %	12·0	—	11·2	12·7
Total fat in dry matter %	33·4	32·7	32·6	36·3
Light petroleum-extractable %	28·4	27·7	27·7	31·1
EtOH-C_6H_6-extractable %	5·0	5·0	4·9	5·2
Light petroleum extract:				
Refractive index (40°)	1·4642	1·4641	1·4642	1·4641
Saponification no.	181	180	181	182
Iodine value (Wijs)	60·5	60·3	62·4	61·3
Hehner number	93·5	92·8	93·8	93·0
Iodine value (fatty acids)	62·3	61·6	63·6	64·8
Phosphorus %	0·337	0·271	0·329	0·251
Nitrogen %	0·160	0·130	0·149	0·119
P/N (atomic)	0·95	0·94	1·00	0·95
Lecithin in extract %	8·7	7·0	8·5	6·5
EtOH-C_6H_6 extract:				
Iodine value (fatty acids)	76·8	79·4	79·3	80·2
Hehner number	69·2	68·4	69·1	66·8
Phosphorus %	1·66	1·79	1·61	1·93
Lecithin in extract %	43·0	46·4	41·7	50·0

Lipoids. The largest yields of fatty material were obtained by the light petroleum extraction and these fractions were examined in detail for the characteristics of the fat. The examination of the second extraction with alcohol-benzene was carried out as far as the weight of extract permitted. Both samples were found to contain lecithin, the second being richer in it than the first. The amounts of lecithin were determined from the phosphorus contents of the fractions and the P/N ratio was determined in all samples. The analyses of the two lipoid fractions are given in Table III.

Food residues from the crop. The presence of traces of food taken in by the parent birds was suspected in the samples. A microscopic examination of the dry matter after staining with aqueous I-KI revealed the presence of starch granules in all samples. The starch was determined by alcoholic precipitation after digestion with alcoholic KOH, hydrolysis with dilute HCl and oxidation with alkaline iodine. The values found varied from 3 to 5% and are given in Table I.

Discussion of results

The dry matter content of the secretion, except for the dried sample, averaged 28·5%, which is between the values given by other workers. The yield of dry matter per bird was very variable, namely, from 0·22 to 0·41 g.

The protein in the samples, at the degree of purification reached, contained from 14·8 to 15·3% of nitrogen. This value is somewhat low owing to the presence of starch. The average N content on a moisture-, ash- and starch-free basis, was 15·6%. Acid hydrolysis showed that the protein was in a fairly pure condition since the humin nitrogen did not exceed 4%.

The nitrogen distribution showed that the protein resembled closely chicken muscle protein. The content of diamino-acids was relatively low and that of the monoamino-acids consequently high. The composition of the protein, derived as it is from tissue protein, thus conformed more closely to that of muscle protein than to that of either egg albumin or blood serum proteins. The protein fraction contained roughly 6% of nitrogen in a non-protein form, or roughly the same amount as in blood serum and cow's milk. The composition of this fraction is probably the same as the "residual" fraction of blood and of other secretions.

The composition of the fat is unique in that it contains considerable quantities of lecithin. Some lecithin was directly extractable from the samples by light petroleum at 40° and a subsequent fraction extracted with an alcohol-benzene mixture was richer in lecithin. This pointed to the presence of lecithin in loose combination with protein in the material, namely, as a lecitho-protein. The behaviour of the lecithin during extraction was similar to that of the lecitho-protein of the protein residue obtained by Rewald [1939] from the extraction of butter fat from butter, in which the lecithin could be removed from protein combination by prolonged extraction with a hot alcohol-benzene mixture. The light petroleum extract contained on the average 7·7% of lecithin calculated from the phosphorus content of the extract, while the alcohol-benzene extract contained 45·3% of lecithin. Acetone precipitated the lecithin in the latter but not in the former fraction. The N/P ratio in the larger fraction was 0·96.

The fat on analysis yielded neither volatile fatty acids nor those of low molecular weight. The iodine value of the fats was 60·3–62·4, and of the fatty acids 61·6–64·8. The fat was of the same degree of unsaturation as goose-fat (58–62) but more saturated than hen fats (79–80) [cf. Hilditch *et al.* 1934]. The fatty acids of the alcohol-benzene fraction, however, showed a higher iodine value (77–80), which for a stearo-oleo-lecithin means 88% of oleic acid in the

fatty acid fraction. The Hehner number for lecithin is 70·5, but the values found for the second fraction were 67–69, which are considered low owing to loss during hydrolysis. The small amount of scum containing solid particles, which usually forms during the ether extraction of acidified, saponified extracts containing lecithin, formed in these cases also and was judged to contribute to the low values obtained for the Hehner number. The petroleum ether extracts behaved normally in this determination, and gave Hehner values comparable with those usually obtained for pure fats.

The mineral matter was characterized by its high K and P contents, medium Ca and low Na and Cl contents. The bulk of the ash consisted of potassium phosphate, as was shown by the readiness with which it fused. P/Ca ratios (atomic equivalents) were 3 or 4 : 1. Appreciable amounts of the P were present in the dried material in forms other than inorganic. Dilute acetic acid extracted only 60–70% of the P in 16 hr., while a cold digestion for 16 hr. with 0·25 *N* KOH extracted only 64–73%. There was no evidence of a phospho-protein present in the secretion.

Generally, the scheme of nutrition provided by the crop secretion favours muscular development, increase in blood volume and bone development. The scheme closely resembles that functioning in the egg before hatching, in that the secretion is devoid of carbohydrates, rich in fat, lecithin, P, K and protein, comparatively rich in Na and Ca and poor in chloride. The secretion bears some resemblance in composition to that of lactose-free dried cow's milk.

Summary

Four samples of crop milk from 115 birds have been investigated in detail. The samples contained 28% dry matter, with 33·8% fat, 58·6% protein, 4·6% ash and 3·9% starch (from an extraneous source) in the dry matter. The protein contained 94% true protein; its nitrogen distribution resembled that of chicken muscle more closely than that of egg or serum proteins.

A light petroleum extract contained 7·7%, and a subsequent alcohol-benzene extract 45·3%, of lecithin, and it appeared as if the lecithin were in loose combination with the protein as lecitho-protein.

The ash was rich in K, Na, Ca and P, and about 30–40% of the P was in organic combination. The secretion was poor in chloride.

The type of food furnished by the crop secretion is very similar to that provided by the egg before hatching.

The author is indebted to Dr S. J. Folley for placing the samples at his disposal.

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CX. THE CARBAMIDO DIACETYL REACTION: A TEST FOR CITRULLINE

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The carbamido-acid citrulline, isolated by Koga & Odake in 1914 from the water melon, attracted no general attention until Wada [1930] established its constitution as α-amino-δ-carbamidovaleric acid, and showed [1933, 1] that it also occurred among the products of the tryptic digestion of caseinogen.

The importance of the acid in nitrogen metabolism was revealed when Krebs & Henseleit [1932] demonstrated that citrulline is an intermediate in the mechanism whereby urea is formed in the liver.

These discoveries raise subsidiary questions regarding the distribution of citrulline in natural proteins, the origin and significance of carbamido-acids, and the nature of the mechanism involving the elaboration of citrulline from its precursors, ornithine, ammonia and carbon dioxide, and its subsequent conversion into arginine. An approach to the study of such problems is begun in the present paper by the introduction of a colour test by which citrulline can be detected in proteins, and its formation traced during a reaction cycle.

Colour reactions of urea and substituted ureas

Since citrulline may be regarded as a substituted urea, a survey was made of urea colour tests applied to proteins. The most familiar of these tests depend on condensation between the urea and an aldehyde in strongly acid solution. The reagents employed include (i) furfuraldehyde [Schiff, 1877], (ii) furfuraldehyde and stannous chloride [Nakashima & Maruaka, 1923], (iii) methylfuril [Fenton, 1903], (iv) *p*-dimethylaminobenzaldehyde [Barrenscheen, 1923].

Although these reagents yield colours with monosubstituted ureas, including citrulline, they are unsatisfactory as tests with most proteins, owing to the presence of tryptophan, which combines with the aldehyde to form pigments that are intensely coloured and completely obscure any reaction given by the citrulline.

A study of the colour reactions between ketones and substituted ureas gave more promising results, for it was observed that diacetyl in strongly acid solution yielded products which, on careful oxidation, were converted into orange or red pigments. Under these conditions tryptophan gave no colour nor did the substituted guanidines, the latter observation indicating that the reactions of diacetyl in acid solution display a selectivity that differs from the reactions in alkaline solution, such as the familiar tests for arginine and creatine, discovered by Harden.

The scope and mechanism of the reactions of diacetyl in alkaline solutions have been investigated by Harden & Norris [1911], O'Meara [1931], and Lang [1932]. O'Meara (personal communication) has observed that diacetyl yields a yellow colour with urea in presence of conc. H_2SO_4, and Smith [1935] has described a similar series of colour reactions between diacetyl and many azides,

including semicarbazide. To avoid confusion, it is proposed to classify these various types of test as follows:

(1) *Carbamido diacetyl reactions*, which occur in acid solution, and are given by compounds containing the system $R.NH.CO.NH_2$, or, in certain instances, $R_1.NH.CO.NH.R_2$.

(2) *Guanidino diacetyl reactions*, which occur in alkaline solution, and are given by compounds containing the system $R.NH.C(:NH).NH_2$, or

$$\begin{matrix} R \\ R' \end{matrix}\!\!>\!N.C(:NH).NH_2.$$

While seeking a stable form of the diketone for use as a class reagent, both the monoxime and the dioxime were examined. The dioxime, as might be expected from its greater stability, was unsatisfactory, but the monoxime proved to be superior in range and sensitivity to ordinary diacetyl. For example, symmetrical dimethylurea yields a deep purple colour with the monoxime reagent, but very little colour with diacetyl itself. In applying the test to substituted ureas, the solution is strongly acidified with HCl, a few drops of 3 % diacetyl monoxime are added, and the mixture is boiled for 3–4 min., during which time an orange colour develops if the test be positive. The colour deepens to red on standing or after addition of a drop of 1 % potassium persulphate.

The scope of the carbamido diacetyl reaction

Using diacetyl monoxime, followed by an oxidizer, the test is positive with: urea, methylurea, butylurea, phenylurea, β-naphthylurea, dimethylurea (symmetrical and asymmetrical), allantoin, semicarbazide, citrulline and all higher proteins examined (ovalbumin, seralbumin, seroglobulin, caseinogen, lactalbumin, fibrin, edestin, gluten, mucin). Commercial peptones and gelatins also give the reaction with varying degrees of intensity.

The test is negative with: ammonium salts, hydrazine, carbamate, cyanate, acetamide, acetylurea, diphenylurea (asymmetric), guanidine, methylguanidine, creatine, creatinine, glycocyamine, uroxamic acid, uric acid, indole and all amino-acids examined, other than citrulline (glycine, glycine ester, sarcosine, alanine, cystine, tyrosine, tryptophan, arginine, histidine, lysine, proline, hydroxyproline, asparagine, aspartic acid).

Reaction with urea. Urea is the only compound so far observed which yields a bright yellow colour, deepening to orange on oxidation. The test is very sensitive, and will reveal 0·1 mg. of urea in 2 ml. of solution, and may be used to show the presence of urea as a contaminant in the monosubstituted ureas, which give a red colour with the reagent. With semicarbazide, the reaction is exceptional in that pigment formation proceeds rapidly in the absence of an external oxidizer, and the compound gradually separates out as a dark red precipitate. Conversely, semicarbazide provides a useful reagent for the detection of diacetyl in aqueous extracts of butter and similar materials.

Reaction with citrulline. The test will give a well-marked red colour with citrulline in concentrations of $M/100$ (0·175 %), and will detect the carbamido-acid in concentrations as low as 0·01 %. The colour changes from madeira to garnet red during oxidation and closely matches the colour given by a tryptic digest of caseinogen, but is redder than the colour given by unhydrolysed caseinogen. It can be matched within ordinary limits by the colour given by pure methylurea or butylurea, uncontaminated by ordinary urea.

The carbamido diacetyl reaction with proteins

About 2 ml. of the protein solution are treated with at least twice as much conc. HCl and 3–5 drops of 3% aqueous diacetyl monoxime. The mixture is boiled for 30 sec., allowed to cool for 2 min., and then carefully oxidized by additon of 1–3 drops of dilute (1%) potassium persulphate or very dilute (0·01%) hydrogen peroxide. A carmine colour develops if citrulline or a similar carbamido-compound be present. The colour deepens on standing or on warming gently. A slight excess of the oxidizer rapidly bleaches the pigment in hot solution.

Notes on the test. (1) H_2SO_4, H_3PO_4 or trichloroacetic acid may be used as condensing agents instead of HCl, but appear to offer no advantages. By adding conc. H_2SO_4 to a mixture of protein and diacetyl monoxime the reaction may be obtained as a "ring" test. In this form it is slow, and several hours are required for colour development at room temperature.

(2) The quantity of acid used should be sufficient to redissolve any protein precipitated by the first addition of acid, previous to heating the mixture.

(3) For maximum colour development, oxidation must be carefully controlled. While almost any of the commoner oxidizing agents may be used, persulphate appears to be the least destructive to the formed pigment. The reaction is inhibited by copper salts, and for this reason is not given by copper citrullinate.

(4) The time of boiling must be curtailed when dealing with proteins rich in carbohydrate residues but relatively poor in citrulline, such as the albumins, or protein preparations containing carbohydrate, such as dried milk powder. When boiled with excess of acid, furfuraldehydes are liberated from the carbohydrate and react with tryptophan in the protein to form violet pigments (Liebermann-Cole reaction). The effect of this accompanying reaction is to change the tint of the citrulline colour from red to purple. Furfuraldehyde pigment formation can be suppressed by avoiding prolonged boiling and the use of excess of protein. Where there is uncertainty, a control test should be carried out in which the diacetyl reagent is omitted. After oxidation, the control test may develop a lilac colour, but neither the intensity nor the shade is comparable with the citrulline colour. When dealing with materials free from tryptophan, such as gelatin, boiling may be continued for several minutes, and the colour allowed to develop spontaneously by atmospheric oxidation. Samples of crude caseinogen prepared from sour milk may contain diacetyl or its precursor, acetylmethylcarbinol (acetoin) in quantities sufficient to give a distinct carbamido-test in the absence of the reagent.

(5) Caseinogen and egg albumin in 1% solutions give a bright purple-red colour with the reagent. Gelatin in 1% solution gives a very feeble reaction unless boiling be continued for at least 5 min. As none of these colours exactly matched the colour given by free citrulline, an endeavour was made to determine approximately the citrulline content of caseinogen by applying the test to a 10% solution that had been subjected to prolonged tryptic digestion. After treatment with charcoal, 1 ml. samples of the digest were submitted to the carbamido-test and the colours compared with that given by a standard ($M/100$) solution of citrulline. Good comparisons were obtained, the colorimetric readings indicating a citrulline value for the caseinogen of the order of 2·0–2·5%.

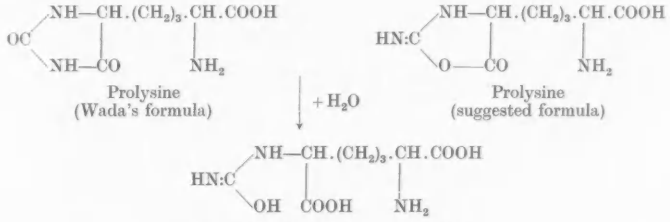
The significance of the carbamido diacetyl reaction with proteins

Citrulline is readily obtained from arginine by alkaline hydrolysis or by bacterial degradation [Ackermann, 1931; Horn, 1933], and its appearance in tryptic digests does not necessarily indicate that it is a preformed component of the original protein molecule, although Wada has claimed that citrulline may be isolated from hydrolysed caseinogen under conditions that do not lead to the conversion of arginine into the carbamido-acid. The present test, it is believed,

affords direct evidence for the existence of citrulline in natural proteins; nevertheless, several possible sources of error must be considered, especially when dealing with proteins hydrolysed in alkaline solution. Some preparations of trypsin, notably commercial pancreatic extracts and autolysed pancreas, are very rich in material giving a positive carbamido-reaction indistinguishable from citrulline, and thus may contribute to the citrulline value of a digest.

Free arginine is readily hydrolysed in alkaline solution (e.g. $N/2$ NaOH) at room temperature, and after a few days yields a gradually intensifying series of carbamido-reactions, and the citrulline value of an apparently sterile tryptic protein digest may increase considerably with time. Lastly, carbamido-acids other than citrulline may be present in the original protein or may arise during hydrolysis. Prolysine, discovered by Wada [1933, 2], is rapidly decomposed by weak alkalis with production of α-amino-α′-carbamidopimelic acid, which gives a positive carbamido-reaction.

Indeed, the instability of prolysine in presence of alkalis is such that one is led to question Wada's formula, and regard the compound as containing an ester linkage rather than the conventional ureide structure.

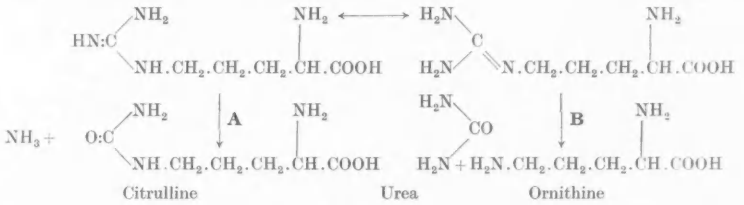


Prolysine, however, is much more stable in acid solutions, and is unlikely to cause a fallacy when the carbamido-test is applied to intact proteins.

A note on the hydrolysis of arginine

The citrulline required for the present work was prepared from arginine by alkaline hydrolysis, according to the method of Fox [1938]. The arginine used was the purest carbonate obtainable from Messrs Schuchardt, and gave no reaction with the carbamido-test. The citrulline was isolated as the copper salt, and was subsequently recrystallized twice. The crystalline carbamido-acid began to melt at 218° and was completely melted at 223° (uncorrected). During trial preparations of citrulline some observations were made on the course of arginine hydrolysis in 0·1 N and 5 N NaOH. The mixtures were boiled under a reflux condenser fitted to an aspirator and acid trap to retain the liberated ammonia. Within the first 15 min. citrulline could be detected by means of the carbamido-test in samples from the mixture. Urea accompanied the citrulline, as was suggested by the colour of the carbamido-reaction, which was yellower in shade than that given by pure citrulline, and the presence of urea was confirmed by the urease test. After 4 hr. hydrolysis in presence of 5N NaOH, the mixture contained in addition cyanate, derived no doubt from the decomposition of urea, and also tetramethylenediamine, derived from the ornithine.

Since urea is not formed from citrulline under these conditions of hydrolysis, it must arise from the arginine directly, the decomposition of which can be represented as following two independent paths.



Reaction **A** is catalysed by the arginine desimidase, described by Horn [1933]. Reaction **B** is catalysed by arginase. During alkaline hydrolysis both reactions occur, but the conditions that determine their relative extents have not yet been elucidated; increase in the concentration of alkali promotes reaction A. For simplicity, the compounds are not represented as zwitterions or as resonance hybrids in the above formulation.

Chemistry of the carbamido-diacetyl reaction

From inspection of the formulae of the reactants, it appears that the test is positive with compounds containing the system $R_1.NH.CO.NH.R_2$, where R_1 is either hydrogen or a simple aliphatic radicle, and R_2 is not an acyl radicle. Thus, phenylurea and symmetrical dimethylurea both form pigments, whereas acetylurea and symmetrical diphenylurea do not. In the absence of an oxidizing agent, diacetyl unites with substituted ureas in acid solution to form cyclic derivatives or diureins investigated by Biltz [1907]. These compounds are colourless and do not yield pigments on oxidation. Their formation involves both ketone groups of the diacetyl. It is suggested that the pigments obtained in the carbamido-reaction are derivatives of a pyrimidine ring, the closure of which is brought about by oxidative condensation involving one methyl group of the diacetyl. The compound obtained from diacetyl and semicarbazide has already been obtained by Thiele [1898], and shown to be a triazine derivative.

Summary

A colour test is described for citrulline and other substituted ureas. The test is capable of being applied directly to proteins, and affords evidence that citrulline exists preformed in caseinogen, egg albumin, and other natural proteins.

Mr E. Kawerau has assisted me in the preparation of the citrulline used in the present work, and I am indebted to Prof. E. A. Werner for specimens of various substituted ureas.

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CXI. IMMUNOLOGICAL AND CHEMICAL PROPERTIES OF CARBOBENZYLOXY-PROTEINS[1]

I. SERUM GLOBULIN AND EGG ALBUMIN DERIVATIVES

II. INSULIN DERIVATIVES

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In previous communications [Hopkins & Wormall, 1933, 1, 2; 1934, 1] the chemical and immunological properties of phenyl- and *p*-bromophenyl-carbamido protein compounds have been described. These compounds were obtained by the condensation of phenyl- and *p*-bromophenyl-*iso*cyanates with various proteins. In other investigations the action of these aryl*iso*cyanates on insulin has been studied [Hopkins & Wormall, 1934, 2; Gaunt & Wormall, 1936; cf. also Jensen & Evans, 1934; 1935].

Bergmann and his colleagues [Bergmann & Zervas, 1932; cf. review by Bergmann, 1933] have shown that benzyl chloroformate readily reacts with the amino group of amino-acids in cooled alkaline solution yielding carbobenzyloxy-amino-acids from which the amino-acid can be regenerated by catalytic reduction with hydrogen in the presence of palladium black. There is no direct evidence that benzyl chloroformate reacts with native proteins in this way, i.e. by condensation with the free amino-groups of the protein molecule; it was thought, however, that if such a reaction occurred, it would be suitable for the investigation of the immunological properties of proteins and also the chemistry of insulin. Preliminary experiments were therefore carried out to see whether benzyl chloroformate reacts with serum globulin, egg albumin and insulin. Bergmann's method was modified slightly, the solution being maintained at *p*H 7·5–8·5 during the reaction in order to minimize the possibility of other changes in the protein molecule taking place. As is described below, this treatment with benzyl chloroformate converts antigenic proteins into acid-precipitable derivatives which have a new antigenic specificity, and in the case of insulin produces a derivative which is devoid of hypoglycaemic power. It was also thought that the new grouping introduced into the protein molecule might be removed by catalytic reduction, with, perhaps, the reproduction of the original immunological specificity in the case of the native proteins and the regeneration of the hypoglycaemic activity in the case of insulin.

[1] The investigations described here were mainly carried out when the authors were members of the Department of Physiology, the University of Leeds.

In the description below, the investigation is divided into two parts, the first part dealing with the preparation and the chemical and immunological properties of carbobenzyloxy-protein compounds, and the second part with the inactivation of insulin by benzyl chloroformate and the chemical and immunological properties of carbobenzyloxy-insulin.

I. SERUM GLOBULIN AND EGG ALBUMIN DERIVATIVES

Preparation of compounds

Carbobenzyloxy-horse serum globulin. The serum globulins were separated from horse serum by means of CO_2 [Hopkins & Wormall, 1933, 1]. 500 ml. of a 2·4% globulin solution were mixed with 400 ml. of phosphate buffer of *p*H 8. The mixture was cooled in ice and 12 ml. of benzyl chloroformate in 50 ml. of ether added slowly to the well-stirred solution. The reaction was maintained at about *p*H 8 by addition of 2*N* NaOH. The globulin compound slowly precipitated out and after 3 hr. the product was centrifuged. The supernatant solution contained no protein and was therefore rejected. The solid material was suspended in water at *p*H 7·5 and extracted 5 times with 30 ml. of ether to remove the excess of benzyl chloroformate. The ether remaining in the suspension was removed by evacuation. The protein material was washed twice with 400 ml. of 0·9% NaCl solution, and the reaction adjusted to *p*H 8·0. Finally the carbobenzyloxy-horse serum globulin was ground in a mortar, strained through fine muslin and suspended in 200 ml. of 0·9% NaCl, the reaction being adjusted to *p*H 7·5.

Carbobenzyloxy-gelatin. 10 g. of gelatin (Coignet's "Gold Label") were dissolved in 300 ml. of water, and 600 ml. of phosphate buffer *p*H 8 and 900 ml. of water were added. The mixture was cooled, 7·5 ml. of benzyl chloroformate in 100 ml. of ether added slowly and the whole mixture was stirred for 3 hr. at room temperature, the reaction being maintained at *p*H 8. At the end of 3 hr. the mixture was centrifuged and the supernatant liquid cooled and acidified with 2*N* HCl to give maximum precipitation of the carbobenzyloxy-gelatin. This product was separated by centrifuging, dissolved in water at *p*H 8·5 by gentle warming to 36–37° and reprecipitated by HCl. After a third precipitation the product was dissolved in the minimum amount of water (180 ml.) and the *p*H adjusted to 7·5.

Carbobenzyloxy-egg albumin. 200 ml. of a 1·7% solution of crystalline egg albumin [Adair & Robinson, 1930] were treated as above with 200 ml. of phosphate buffer of *p*H 8 and 7·5 g. of benzyl chloroformate in 30 ml. of ether. The mixture was stirred at 5° for 3 hr. and then centrifuged. The supernatant solution was acidified with dilute acetic acid to give maximum precipitation of the carbobenzyloxy-egg albumin. This protein derivative was then separated by centrifuging and redissolved in water by adding 2*N* NaOH to give *p*H 8. The solution was centrifuged and the protein derivative in the supernatant solution was precipitated by dilute HCl. After two further precipitations the carbobenzyloxy-egg albumin was dissolved in 0·9% NaCl at *p*H 7·5. A small amount of chloroform was added as a preservative to the solutions of the carbobenzyloxy derivatives of gelatin and egg albumin.

Carbobenzyloxy-amino-acids. For the purposes of inhibition tests a few carbobenzyloxy-amino-acids were made. The method described by Bergmann &

Zervas [1932] for the synthesis of peptides has been used so widely that the carbobenzyloxy-derivatives of most of the naturally occurring amino-acids have been prepared, usually by addition of benzyl chloroformate to a cooled aqueous solution of the amino-acid in the presence of a considerable excess of alkali. For the purposes of the present research it was thought advisable to reproduce in these preparations the conditions obtaining in the production of the carbobenzyloxy-protein derivatives, i.e. to carry out the reaction in the presence of phosphate buffer at *p*H 8.

Immunization

The carbobenzyloxy-horse serum globulin was prepared as described above and the suspension kept in the ice chest, phenol being added to give a concentration of 0·25% in the suspension. The suspension was injected intraperitoneally into four rabbits at intervals of 7–9 days, each rabbit receiving at each injection a suspension containing 0·25 g. of the protein. The sera were tested 7 days after the third and fifth injections. One rabbit gave a good antiserum after three injections; the others required two further injections.

Precipitin tests

Precipitin tests were carried out as previously described [Hopkins & Wormall, 1933, 1]. Readings were taken after $\frac{1}{2}$, 1 and 2 hr. at 37° and usually after a further 18 hr. at room temperature; those recorded in Tables I and II were obtained after 1 hr. In the case of carbobenzyloxy-gelatin compounds the tests were made at room temperature (15–19°), more pronounced precipitates being obtained in this way [Hopkins & Wormall, 1933, 2].

The extent of precipitin formation is recorded as follows (in increasing degrees of precipitation):

− (no reaction), f.tr. (faint trace), tr. (trace), ±, +, + ±, + +.

Inhibition tests

Precipitin inhibition tests were carried out under the conditions previously described [Hopkins & Wormall, 1934, 1], the inhibiting substance being in neutralized 0·01 *M* solution. The degree of precipitin formation was noted immediately and after 5 min., $\frac{1}{2}$ and 1 hr. These inhibition tests were carried out at room temperature when carbobenzyloxy-gelatin was used as the antigen, and at 37° in other cases.

The immunological properties of carbobenzyloxy-proteins

The sera obtained by the injection of carbobenzyloxy-horse serum globulin into rabbits were tested against horse serum, carbobenzyloxy-egg albumin and carbobenzyloxy-gelatin. Marked reactions were obtained with the last two antigens (cf. Table I), indicating the possession of good antigenic power by the carbobenzyloxy-globulin. With horse serum the reactions were either feeble or negative, indicating almost complete loss of the original species specificity. The precipitin reactions with carbobenzyloxy-egg albumin and carbobenzyloxy-gelatin show that an immunological relationship exists between carbobenzyloxy-horse serum globulin and the corresponding derivatives of widely differing proteins. This relationship has been undoubtedly conferred on these proteins (e.g. gelatin and egg albumin) by the introduction of the carbobenzyloxy-grouping. The reactions with the gelatin compound are more strongly marked than those with the egg albumin compound. This is particularly interesting since gelatin itself is non-antigenic, whereas egg albumin is a true antigen.

In order to obtain confirmation of the view that the reaction between benzyl chloroformate and proteins involves the free amino groups of the protein molecule a few inhibition tests have been carried out. The results given in Table II show that carbobenzyloxy-glycine inhibits the reaction between carbobenzyloxy-proteins and the antisera to carbobenzyloxy-horse serum globulin, whereas it has no influence at all on the formation of precipitates in other antigen-antibody reactions (e.g. the horse serum anti-horse serum precipitin reaction). The carbobenzyloxy-protein precipitin reaction is partially inhibited by phenyl-carbamido-glycine.

Table I. *Precipitin reactions between carbobenzyloxy-protein derivatives and antisera to carbobenzyloxy-horse serum globulin*

Antigen	Dilution of antigen	Immune serum against carbobenzyloxy-horse serum globulin		
		No. 420	No. 421	No. 422
Horse serum	1:20	tr.	—	f.tr.
	1:100	tr.	tr.	f.tr.
	1:500	±	tr.	±
	1:2500	tr.	–	tr.
Carbobenzyloxy-egg albumin	1:20	f.tr.	–	f.tr.
	1:100	±	+	tr.
	1:500	±	+	+
	1:2500	tr.	±	±
Carbobenzyloxy-gelatin	1:20	tr.	f.tr.	tr.
	1:100	+	+	+
	1:500	+ ±	+ ±	+ ±
	1:2500	tr.	±	±

The dilutions of antigen refer to dilution of a solution which contains 5% of total protein.

Table II. *Inhibition of the precipitin reactions*

Antigen	Antiserum against		"Inhibiting" substance			
			NaCl	Glycine or alanine	Carbobenzyloxy-glycine (or alanine)	Phenyl-carbamido-glycine
Horse serum	Horse serum		+ +	+ +	+ +	+ +
Carbobenzyloxy-gelatin	Carbobenzyloxy-horse serum globulin	(*a*)	+ ±	+ ±	–	+
		(*b*)	+	+	–	±

(*a*) and (*b*) give the results with different antisera.

II. INSULIN DERIVATIVES

The effect on insulin of chemical reagents which possess a more or less specific action on certain groups present in proteins has been studied by many authors. By these methods a considerable amount of information has been obtained regarding the groupings in the insulin molecule which may be responsible for its hypoglycaemic activity. A few of these methods which result in the inactivation of the hormone are reversible, i.e. the activity of the original insulin can, at least in part, be regenerated under suitable conditions. Partial reactivation can be effected in insulin inactivated by acetic anhydride, formaldehyde or hot dilute acid [for a review of the literature cf. Freudenberg *et al.* 1930; 1931; Jensen & Evans, 1934], by acid-alcohol [Carr *et al.* 1929; Charles & Scott, 1931],

and by mild reducing agents such as cysteine and glutathione [Freudenberg & Wegmann, 1935]. Perhaps the most satisfactory reactivation of this type is, however, that obtained by Harington & Neuberger [1936]. These authors have shown that iodination of insulin, resulting in the substitution of iodine in the 3:5-positions of the tyrosine groupings, causes a 90–95% loss of activity. Partial removal of the iodine by catalytic reduction is accompanied by an approximately proportional restoration of the hypoglycaemic activity.

For the reasons stated in the first part of this paper, it seemed likely that benzyl chloroformate would combine with the insulin molecule, possibly with loss of the hypoglycaemic activity of the hormone. Furthermore, it was thought that, if inactivation occurred, it might be found to be reversible, since the carbobenzyloxy-groupings attached to amino-acids can be removed by catalytic reduction. Preliminary experiments showed that benzyl chloroformate reacts with insulin and the properties of the product of this reaction (carbobenzyloxy-insulin) have been studied. A brief report of these investigations has been made [Gaunt *et al.* 1935].

Experimental

The insulin activities of the original material and the treated insulin were determined as previously described [Hopkins & Wormall, 1934, 2]. In practically all the experiments, tests were made with groups containing four rabbits. Duplicate sugar determinations (0·2 ml. blood for each) were carried out with every sample of blood from each rabbit, the method of Hagedorn & Jensen [1923] being used. In the tables the average value for each group is given.

The reaction between benzyl chloroformate and insulin was allowed to proceed at 5–8° and in the presence of 0·2 *M* $NaHCO_3$ at *p*H 8–8·5. The use of bicarbonate instead of the phosphate buffer of *p*H 8 is the only modification of the method employed in the experiments with native proteins as described in the previous part of this paper. The insulin used for this work contained 19,500 units per g.

In all experiments a portion of the insulin solution was removed, before any treatment, for use as a control. This control was usually subjected to the same treatment as the experimental sample without, of course, the addition of benzyl chloroformate. The various solutions injected into the rabbits were treated with phenol (to give 0·25%).

The action of benzyl chloroformate on insulin

32·8 mg. of insulin were dissolved in 4 ml. of 0·9% NaCl and 4 ml. of 0·2 *M* $NaHCO_3$, 2 drops of 0·2 *N* NaOH being added to give a clear solution at *p*H 8–9; 0·5 ml. of this solution was removed for use as a control in the physiological assay. The remainder of the insulin solution was cooled in ice and treated with 0·8 ml. of benzyl chloroformate, the reaction being maintained at *p*H 8 by addition of 0·2 *N* NaOH when necessary. After the mixture had been shaken for 2 hr., the excess of benzyl chloroformate was removed by shaking eight times with 10 ml. ether at each extraction. The residual ether was removed by gentle warming and evacuation. The suspension of carbobenzyloxy-insulin was injected in such amounts that each rabbit received per kg. the treated material from 5 to 15 units of insulin. From the results of the blood sugar determinations it could be concluded that probably all the activity of the insulin had been destroyed as a result of treatment with benzyl chloroformate (cf. Table III).

As the insulin compound is insoluble it was thought advisable to determine whether the inability of carbobenzyloxy-insulin to lower the blood sugar of starved rabbits was due simply to its insolubility. If this is true, then the

administration of large amounts of insulin compound might exert a slow effect after injection. Blood sugar determinations carried out up to about 24 hr. after the injection indicate, however, that the carbobenzyloxy-insulin preparations have no power to reduce the blood sugar even when observations are made over a long period of time.

Table III. *Inactivation of insulin by benzyl chloroformate*

Group	Subcutaneous injection of	No. of units per kg.	Blood sugar (mg./100 ml.) Time after injection (hr.) 0	$2\frac{1}{2}$	$4\frac{1}{2}$	$5\frac{1}{2}$	$23\frac{1}{2}$
A	Insulin	0·75	82	49	55	70	107
B	Carbobenzyloxy-insulin	15	91	97	99	106	96

The rate of inactivation of insulin by benzyl chloroformate

Experiments have been carried out to determine the rapidity with which benzyl chloroformate inactivates insulin. A solution of 41 mg. of insulin in 0·9% NaCl and 0·2 *M* $NaHCO_3$ was cooled in ice and treated with 0·5 ml. of benzyl chloroformate, the mixture being well shaken. Samples were removed at intervals of 5, 30 and 60 min. These samples, and the control sample removed before addition of the reagent, were made slightly acid and immediately extracted six times with about 10 ml. portions of ether. The remaining ether was removed by gentle warming and evacuation. The 5, 30 and 60 min. samples, injected into rabbits in amounts equivalent to 4, 10 and 15 units of the original insulin respectively, showed no significant insulin activity (cf. Table IV). The results of this experiment indicate that the inactivation of insulin by benzyl chloroformate is rapid, though it is probable that the results obtained are only of qualitative value, as it is difficult to ensure complete removal of the excess of benzyl chloroformate by extraction with ether.

Table IV. *Time required for inactivation of insulin by benzyl chloroformate*

Preparation		No. of units per kg.	Blood sugar (mg./100 ml.) Time after injection (hr.) 0	$1\frac{1}{2}$	$3\frac{1}{2}$	$5\frac{1}{2}$
Insulin		0·75	103	50	46	47
Carbobenzyloxy-insulin	5 min.*	4	103	99	94	97
	30 min.*	10	104	101	98	96
	60 min.*	15	94	103	100	101

* These samples were obtained by allowing the benzyl chloroformate to act on the insulin for 5, 30 and 60 min. (see text for full details).

The effect of small amounts of benzyl chloroformate on insulin

It is possible that total inactivation of insulin may occur when only a few of the free amino-groups of the insulin molecule have been blocked by benzyl chloroformate. In order to test this hypothesis experiments were carried out to determine the minimum amount of benzyl chloroformate required to effect this inactivation. For the purposes of these experiments the amounts are expressed in terms of that needed to combine with all the amino-groups of the insulin molecule, based on the assumption that insulin contains 1% of amino-nitrogen.

In the first experiment it was found that 12·5 times the theoretical amount of benzyl chloroformate inactivated insulin completely. In a second experiment of a similar type approximately 5, 3 and 2 times the theoretical amounts of

benzyl chloroformate were allowed to react with insulin. The blood sugar values obtained after the injection of the products of these reactions into starved rabbits are given in Table V. From these figures it will be seen that 5 and 3 times the theoretical amounts of benzyl chloroformate cause complete inactivation of insulin, and twice the theoretical amount destroys at least 95% of the original hypoglycaemic activity of the hormone.

Further experiments were carried out using the theoretical and half-theoretical amounts of benzyl chloroformate. The results indicate that with the theoretical quantity about 60% of the original activity is retained, whilst with half the theoretical amount of the reagent over 80% of this activity remains (Table VI).

Table V. *Effect of different amounts of benzyl chloroformate on insulin*

Amount of benzyl chloroformate allowed to react with insulin	Amount of product injected ("units" per kg.)	Blood sugar (mg./100 ml.) Time after injection (hr.)		
		0	1¼	4¼
0	0·75	103	57	88
2 × theoretical	2·75	94	92	85
2 × ,,	0·75	91	97	104
3 × ,,	0·75	91	100	107
5 × ,,	1·5	95	100	109

Experimental details. 32·5 mg. of insulin were dissolved in 4 ml. of 0·9% NaCl and 4 ml. of 0·2 *M* $NaHCO_3$ at *p*H 8–8·5. The solution was divided into 4 lots each of 2 ml., all the samples being then cooled in ice. To three of these samples were added 0·96 ml., 0·58 ml. and 0·38 ml. of a 0·5% solution of benzyl chloroformate in ether. The volume of ether in all four samples was made up to 0·96 ml. The samples were shaken for 2 hr. and then left overnight in the ice chest. The ether was removed by warming and evacuation. The *p*H of the solutions did not alter during the course of the reaction. After suitable dilution with 0·9% NaCl the samples were injected into rabbits.

Table VI

Amount of benzyl chloroformate allowed to react with insulin	Amount injected ("units" per kg.)	Blood sugar (mg./100 ml.) Time after injection (hr.)		
		0	1½	6
0	0·6	105	51	68
0	0·3	99	69	97
Theoretical amount	0·6	96	66	78
Theoretical amount	0·3	92	67	78
0·5 × theoretical amount	0·6	88	45	55
0·5 × theoretical amount	0·3	86	62	78

Experimental details. Similar to those for Table V, with smaller amounts of benzyl chloroformate.

Attempts to reduce carbobenzyloxy-proteins catalytically

In view of the ease with which the carbobenzyloxy-group can be removed from carbobenzyloxy-amino-acids by catalytic reduction [Bergmann & Zervas, 1932], it was decided to attempt a similar reduction with the carbobenzyloxy-derivatives of insulin and other proteins. Preliminary experiments were first made to determine the effect of catalytic reduction on untreated insulin. Treatment with hydrogen in the presence of palladium black was found to effect in some experiments, but not in all, appreciable inactivation of insulin. The palladium black undoubtedly adsorbs some of the insulin, but this adsorption (measured by total N determinations on the solutions) was not always sufficient to account for the loss of activity. Freudenberg *et al.* [1931] also experienced this adsorption in their efforts to reduce insulin catalytically, and were unable to overcome the difficulty. Since the experiments described here were completed, Harington & Neuberger [1936] have effected the removal of about two-thirds of

the iodine of iodinated insulin by catalytic reduction with a palladium-barium sulphate catalyst in pyridine-water solution and an atmosphere of hydrogen; this reduction does not appear to have any destructive action on the physiologically active groups of insulin.

In view of the difficulties experienced with insulin, further experiments were made with carbobenzyloxy-gelatin. No significant increase in free amino-N occurred, however, when this derivative was exposed in solution at *p*H 7 to the action of palladium black and hydrogen for several hours. It was concluded that some other method would have to be devised for the removal of the carbobenzyloxy-groups and in the case of the insulin derivative care would have to be taken to prevent loss of physiological activity due to reduction of the S—S groups.

Since the work described in this paper was completed, Clutton *et al.* [1937] and Clutton *et al.* [1938] have succeeded in removing the carbobenzyloxy-grouping from *O*-β-glucosido-*N*-carbobenzyloxytyrosyl protein compounds. In the case of the gelatin compound it was found impossible to effect this removal by reduction in aqueous solution with a variety of catalysts and reducing agents. However, by reduction with sodium in liquid ammonia the carbobenzyloxy-grouping was removed from the *O*-β-glucosido-gelatin derivative without rupture of the glucosido-linkage and without degradation of the protein. This method, however, could not be used in the case of *O*-β-glucosido-*N*-carbobenzyloxytyrosyl horse serum globulin; here removal of the carbobenzyloxy-grouping was effected by reduction at *p*H 9·6 in an atmosphere of hydrogen in the presence of palladium black. These workers did not attempt to remove the carbobenzyloxy-grouping from *O*-β-glucosido-N-carbobenzyloxytyrosyl insulin and little information is still available regarding the influence of such reducing agents on the insulin molecule.

Numerous authors have observed allergic reactions following the injection of insulin into man, and although in most instances the reactions observed can be attributed to other proteins present in the insulin preparations, there is evidence that pure insulin occasionally produces an allergic response. It is not certain that this hypersensitivity is due to a true antigen-antibody reaction, but the blood of hypersensitive patients has been shown to contain precipitins to insulin [Raynaud & LaCroix, 1925; Karr *et al.* 1933]. Stronger evidence in support of the view that insulin can function as an antigen has been obtained by Lewis [1937]. This author injected relatively large doses of insulin into virgin guinea-pigs, controlling the hypoglycaemia by the administration of glucose, and found that the uteri of these sensitized animals reacted with insulin but not with the pancreas protein or with the serum of the animal from which the insulin was derived. Cross-reactions with hog and beef insulins indicated that insulins from different animal species are immunologically closely related.

The injection of large amounts of insulin into an animal for the production of antibodies is precluded by the intense hypoglycaemic effect of the hormone, although the hypoglycaemia can be partially controlled by the administration of glucose. An alternative method is to inactivate those groups which are responsible for the hypoglycaemic activity by some process which does not destroy the antigenic power of proteins; this inactivated product could then be injected in amounts which are normally needed to incite antibody response to antigenic proteins. From evidence produced in this paper benzyl chloroformate appears to be suitable for this purpose, since it does not appreciably reduce the antigenic power of serum globulins and yet it rapidly destroys the hypoglycaemic activity of insulin. An experiment has therefore been carried out to determine whether

the injection of large amounts of carbobenzyloxy-insulin produces in mice any change in the sensitivity of the animals towards insulin, and whether any serological evidence of the formation of an anti-insulin can be obtained.

A group of 40 mice was given a series of injections of carbobenzyloxy-insulin and the animals subsequently tested for their response to insulin, and their serum for antibodies to carbobenzyloxy-insulin.

0·4 g. of insulin was treated as described previously with five times the "theoretical" amount of benzyl chloroformate, and the final product diluted to 56 ml. with the addition of NaCl (to give 0·9%) and phenol (0·2%): 0·25 ml. of this solution contained the equivalent of 1·8 mg. of insulin. Each mouse received four intraperitoneal injections, each of 0·0125 ml. of this solution per g., at intervals of 2–3 days. During the period of immunization the mice were fed on bread soaked in 10% glucose solution and were given a strong solution of glucose to drink in order to counteract the slight hypoglycaemic action of the carbobenzyloxy-insulin. Any mouse which showed signs of hypoglycaemia was given a subcutaneous injection of 0·25–0·5 ml. of sterile 10% glucose, but in spite of this treatment a few of these mice (and all those which received similar injections of phenylcarbamido-insulin) died during the immunization. A similar control group of mice received injections of a solution containing 0·9% NaCl and 0·2% phenol. At various periods after the last injection (4, 9 and 17 days), these two groups of mice and another group of untreated mice were tested for their response to insulin. The technique employed was that of Trevan & Boock [1926]; the mice received subcutaneous injections of 0·015 (or 0·030) unit per 20 g. and were kept at 38° during the assay. No significant difference between the three groups was observed, and the conclusion was reached that injections of large amounts of carbobenzyloxy-insulin had not led to any detectable change in the response of these mice to subsequent insulin treatment.

The serum of the mice "immunized" against carbobenzyloxy-insulin was tested, in complement-fixation and precipitin tests, against carbobenzyloxy-insulin and carbobenzyloxy-gelatin. The results were all negative.

Discussion

The reaction between benzyl chloroformate and proteins appears to take place mainly if not entirely between the reagent and the free amino-groups of the protein. The reaction is accompanied by the total disappearance of free amino-N, as determined by "formol" titration, and the blocking of the amino-groups is apparently complete. This view is supported by the results of the immunological investigations, for the reaction between carbobenzyloxy-proteins and their antisera is completely inhibited by carbobenzyloxy-amino-acids. To a lesser degree, phenylcarbamido-amino-acids inhibit this precipitin reaction, and this can be attributed to the structural relationship between the carbobenzyloxy- and phenylcarbamido-amino-acid groups. Cross-reactions of this type are quite frequent in serological inhibition tests [cf. Landsteiner, 1936, pp. 122–32].

$$\underset{\text{carbobenzyloxy-}}{>CH.NH.CO.O.CH_2.C_6H_5} \qquad \underset{\text{Phenylcarbamido-}}{>CH.NH.CO.NH.C_6H_5}$$

The results of the immunological experiments described here provide further evidence for the view that substitution in the free amino-groups of antigenic proteins leads to complete, or almost complete, loss of the original specificity and to the acquirement of a new specificity which is characteristic for the introduced grouping. It seems possible that the introduction of any new

group will cause this radical change in the immunological properties of a protein if the new group is sufficiently prominent [cf. Landsteiner, 1936]. The relatively small size of the methylene group might account for the retention of species specificity in formaldehyde-treated proteins; on the other hand, this retention might be due to incompleteness of the reaction between formaldehyde and the free amino-groups, for there is not universal agreement as to the precise nature of this reaction [cf. review by Jordan Lloyd & Shore, 1938].

The action of benzyl chloroformate on insulin also appears to involve the free amino-groups of the protein. The reaction is accompanied by practically complete loss of the hypoglycaemic power of the hormone, similar to, but possibly more complete than, that effected by phenyl *iso*cyanate [Hopkins & Wormall, 1934, 2]. The minimum amount of benzyl chloroformate needed for complete inactivation is about twice the amount required by theory to react with all the free amino-groups. The fact that the "theoretical" amount of the reagent does not effect complete inactivation might be due to action of the reagent on groups other than amino-groups, or, more probably, to loss of the acid chloride by the action of water. The inactivation of insulin is very rapid and takes place at *p*H 8–8·5 and at ordinary temperatures. The results offer strong support for the view that the free amino-groups of insulin are of significance in connexion with its physiological activity [Hopkins & Wormall, 1934, 2; Jensen & Evans, 1934; 1935].

The attempts to "immunize" mice with carbobenzyloxy-insulin were made in order to determine (*a*) whether the injection of large amounts of this insulin derivative would influence the response of the animals to ordinary insulin, and (*b*) whether carbobenzyloxy-insulin would behave as a full antigen. Immunological investigations on insulin are complicated by the hypoglycaemic action of this substance, but this difficulty is overcome by the use of carbobenzyloxy-insulin, which, by analogy with other proteins, should be antigenic if insulin itself has this function. Any antibodies to the inactivated insulin might be expected to show some capacity to react with unchanged insulin, since antisera to carbobenzyloxy-globulin usually give slight precipitation with the untreated globulin. In the experiments described here there was no indication of antibody formation of this type. The sera of the injected mice contained no detectable antibodies to the carbobenzyloxy-derivatives of insulin or gelatin, and furthermore, these animals showed no abnormality when injected with amounts of insulin which are usually sufficient to produce convulsions. It is not easy to forecast exactly what will happen in an animal if an anti-insulin is produced. Small amounts of the antibody could presumably be "neutralized" by insulin with the formation of a complex which might possess some hypoglycaemic activity, but excess of the antibody might be expected to render the animal diabetic. If the animal survives the immunization, perhaps the only evidence of the formation of an anti-insulin might be a change in the response of the animal to insulin injection. Further information on this point might throw some light on problems of insulin-hypersensitivity and insulin-resistance in man.

The antigenic properties of hormones have been studied by numerous authors during recent years, largely stimulated by the observations of Collip and his colleagues. As far as insulin is concerned, the available evidence is rather conflicting. The previously mentioned work of Lewis [1937] suggests that sensitization to insulin can be produced, and Barral [1935] claims to have produced an antigenic and non-hypoglycaemic "aninsulin" by the action of formaldehyde on insulin for 40 days at 60°. Spaccarelli [1936], on the other hand, found that the uteri of insulin-treated virgin guinea-pigs do not react with

insulin, although the uteri of animals injected with a mixture of serum and insulin react to insulin alone. Interesting observations on the antigenicity of insulin have recently been made by Clutton *et al.* [1938]. These workers immunized rabbits against *O*-β-glucosido-*N*-carbobenzyloxytyrosyl insulin and found that although the antisera produced to the insulin derivative failed to precipitate the homologous antigen, they gave precipitates with *O*-β-glucosido-*N*-carbobenzyloxytyrosyl horse serum globulin. Further, the antisera to the globulin derivative gave precipitates with the insulin compound. The fact that the insulin derivative inhibited the cross reaction of its antiserum with the globulin derivative indicated that, despite the failure of the insulin derivative to precipitate its antiserum, a definite antigen-antibody reaction does occur. These authors suggest that the physical properties of the antigen may be such that an insoluble antigen-antibody complex cannot be formed. They conclude that coupling with glucosidotyrosine converts insulin into a full antigen. The question of the antigenicity of insulin itself is, however, still undecided, and, as far as observations on individuals hypersensitive to insulin are concerned, it might be fair to say that they do not as yet offer conclusive evidence of the presence in the blood of true antibodies to pure insulin.

Summary

1. Benzyl chloroformate, the reagent used by Bergmann for the preparation of carbobenzyloxy-amino-acids, readily acts on serum globulin, gelatin and other proteins, at ordinary temperatures and at *p*H 7·5–8·5. The reaction appears to be concerned mainly with the free amino-groups of the proteins.

2. The injection of carbobenzyloxy-serum globulin into rabbits leads to the production of antibodies which react with the carbobenzyloxy-derivatives of heterologous proteins. The original species-specificity of the serum globulin is almost completely destroyed.

3. The reaction between carbobenzyloxy-proteins and their antisera is completely inhibited by carbobenzyloxy-amino-acids. The immunologically characteristic groups of such proteins are, therefore, the carbobenzyloxy-amino-groups (—NH.CO.O.CH_2.C_6H_5).

4. Benzyl chloroformate rapidly destroys the hypoglycaemic activity of insulin. The amount of the acid chloride needed to effect complete inactivation is about twice the amount required by theory to react with all the free amino-groups of insulin, although smaller amounts produce appreciable inactivation. These observations support the view that the free amino-groups of insulin are important for its hypoglycaemic activity.

5. Mice injected with relatively large amounts of carbobenzyloxy-insulin behaved exactly like the control animals when injected with a "convulsive" dose of insulin. These "immunized" mice showed no significant hypo- or hypersensitivity to insulin, and their sera contained no detectable antibodies to carbobenzyloxy-insulin or carbobenzyloxy-gelatin.

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CXII. A METHOD FOR THE ESTIMATION OF "TRUE" SUGAR IN 0·05 ml. OF BLOOD

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A method which gives most nearly the true glucose values for blood should show the following characteristics:

(i) Glutathione and other non-sugar reducing substances are excluded either by the protein precipitants [Somogyi, 1929; 1930] or by using unhaemolysed blood [Herbert & Bourne, 1929; 1930].

(ii) The sugar values obtained are identical with the amounts of sugar fermentable by yeast, and the entire blood sugar so determined is fermentable.

(iii) Good results are obtained when pure glucose is estimated in solution and after addition to blood.

A titration method meeting these requirements was recently described [King *et al.* 1937] for 0·2 ml. of blood. A modification of this method is here described in which the blood sample is 0·05 ml. The protein precipitants used are sodium tungstate and copper sulphate [Somogyi, 1931] and the filtrate is treated with a modified Harding & Downs [1933] copper reagent. The cuprous oxide formed is estimated by the colour produced with a phosphomolybdic acid solution [Folin & Wu, 1929].

With this method, glucose was estimated accurately in pure solutions and after addition to blood. Determinations were made using pure glucose solutions, normal bloods, diabetic bloods and mixtures of these bloods with glucose solutions. The results obtained were identical with those found using the previously described method [King *et al.* 1937].

Experimental

Technique of the analyses

(*a*) *Pure glucose solutions.* The glucose solution (1 ml.) was mixed with 1 ml. of the copper reagent in a wide test tube ($\frac{3}{4}$ in. diameter). The tube, stoppered with cotton-wool, was placed in a boiling water bath for exactly 10 min. After immediate cooling, the mixture was treated with the phosphomolybdic acid (3 ml.). The colours were read within 15 min. in a Duboscq type colorimeter fitted with an Osram sodium light.

Glucose solutions of concentrations from 0 to 75μg./ml. (equivalent to from 0 to 300 mg./100 ml. in blood at a 1/40 dilution) were treated as above.

The colorimetric readings are shown in Table I.

Glucose standards in benzoic acid gave identical readings.

(*b*) *Application to blood.* 1 ml. of blood filtrate (1/40 dilution) was used exactly as the glucose solutions above. The method is as follows. 0·05 ml. of whole blood is pipetted into 1·75 ml. of isotonic sodium sulphate solution, or isotonic sodium sulphate fluoride-thymol solution. 0·1 ml. each of sodium tungstate and of copper sulphate are added and the solutions thoroughly mixed. The mixture is centrifuged. 1 ml. of the filtrate (=0·025 ml. of blood) is mixed with 1 ml. of the

Table I

μg. of glucose per ml. of "test" solution	Reading (mm.) of 20 μg./ml. "standard" solution	Reading (mm.) of 50 μg./ml. "standard" solution
0	0	—
5	2·5	—
10	5·0	—
15	7·5	—
25	12·5	—
30	15·0	6·0
35	17·5	—
40	—	8·0
50	25·1	—
60	—	12·1
75	—	15·1

mixed copper reagent in a wide test tube ($\frac{3}{4}$ in. diameter). The tube, stoppered with cotton-wool, is placed in a boiling water bath for exactly 10 min. After immediate cooling, 3 ml. of the phosphomolybdic acid reagent are added. The colour produced is compared with that produced by 1 ml. of a standard glucose solution in benzoic acid, treated in the same way as the blood filtrate.

Calculation. Let x = concentration of standard in μg./ml.

$$\text{Blood sugar (mg./100 ml.)} = \frac{\text{reading of standard}}{\text{reading of test}} \times \frac{x}{1000} \times \frac{100}{0{\cdot}025};$$

e.g. with 20 μg./ml. standard

$$\text{Blood sugar (mg./100 ml.)} = \frac{\text{reading of standard}}{\text{reading of test}} \times \frac{20}{1000} \times \frac{100}{0{\cdot}025}$$

$$= \frac{\text{reading of standard}}{\text{reading of test}} \times 80.$$

Similarly, with 50 μg./ml. standard

$$\text{Blood sugar (mg./100 ml.)} = \frac{\text{reading of standard}}{\text{reading of test}} \times 200.$$

Table II shows a comparison of the titrimetric method [King *et al.* 1937] and the present method. Determinations by both methods were carried out independently.

Table II. *Blood sugar (mg./100 ml.)*

Titrimetric method	Colorimetric method
85	84
85	84
90	89
90	89
72	74
73	74
77	78
103	101
103	101
130	129
130	130
108	109
129	128
164	165
189	186
240	242
216	217
300	302

(*c*) *Recovery experiments.* These were carried out using both methods, the amount of glucose added in both cases being proportional.

	Quantities (ml.) used	
	Titrimetric method	New colorimetric method
Blood	0·2	0·05
Sodium sulphate	1·2	1·25
Glucose solution in isotonic sodium sulphate	2·0	0·5
Copper sulphate	0·3	0·1
Sodium tungstate	0·3	0·1

The results obtained are shown in Table III.

Table III. *Blood sugar (mg./100 ml.)*

Added equivalent of 50 mg./100 ml. glucose		Added equivalent of 100 mg./100 ml. glucose		Added equivalent of 200 mg./100 ml. glucose	
Titrimetric method	Colorimetric method	Titrimetric method	Colorimetric method	Titrimetric method	Colorimetric method
127	129	103	101	85	86
178	178	203	202	289	291
63	64	67	66	107	108
114	114	165	167	306	309
105	104	—	—	67	67
153	154	—	—	267	266

Solutions

(1) *Copper reagent. Solution A.* 13 g. $CuSO_4$, $5H_2O$ are dissolved in water and the volume made up to 1 l.

Solution B. 50·0 g. $NaHCO_3$ are dissolved by shaking in the minimum amount of distilled water. When all the bicarbonate is dissolved, 40·0 g. anhydrous Na_2CO_3 are added to the solution and the mixture shaken until all the carbonate has dissolved. 36·8 g. potassium oxalate are dissolved in successive small quantities of warm water and added to the mixture. Finally a solution of 24·0 g. sodium potassium tartrate in the minimum amount of water is added to the mixture. The volume is made up to 1 l. and the mixture well shaken.

The copper reagent used is prepared freshly each day and is a mixture of exactly equal volumes of solutions A and B.

(2) *Phosphomolybdic acid reagent* [Folin, ~~1930~~]. 35 g. molybdic acid and 5 g. sodium tungstate are dissolved in 250 ml. of 0·2 *N* NaOH and boiled for 30 min. Water is added to about 350 ml., 125 ml. 89% H_3PO_4 (sp. gr. 1·75) are added, and the volume made up to 500 ml.

(3) *Isotonic sodium sulphate containing fluoride and thymol to prevent glycolysis.* 100 mg. NaF and 10 mg. thymol are dissolved in 100 ml. of isotonic Na_2SO_4 (3 g. Na_2SO_4, $10H_2O$/100 ml. solution).

(4) *Sodium tungstate* (10 g./100 ml.).

(5) *Copper sulphate* (7 g. $CuSO_4$, $5H_2O$/100 ml.).

(6) *Stock glucose solution.* 0·1 g. pure glucose is dissolved in saturated benzoic acid solution and the volume made up to 100 ml. This is a permanent standard.

(7) *Standard glucose solutions.* These are prepared by diluting 2, 3, 4, 5, etc. ml., to 100 ml. with saturated benzoic acid solution; giving standards of 20, 30, 40, 50, etc., μg./ml.

SUMMARY

A colorimetric modification (applicable to 0·05 ml. of blood) of a previously described titrimetric copper method for the determination of blood sugar is described.

The method is believed to give true glucose values.

Estimations on normal and diabetic blood by the new method, and also by the previously described method, give results which are in agreement.

The authors wish to express their thanks to Dr E. J. King for his continual advice and encouragement, and also acknowledge previous preliminary experiments by Dr T. F. Nicholson and Dr Y. Seltzer in this laboratory.

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CXIII. THE PRECIPITATION OF PROTEINS WITH COMPLEX SALTS

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Attention is directed to a group of protein precipitants which are of interest both from a practical and theoretical point of view. The commonly used protein precipitants are subject to certain limitations. Some of them, such as phosphotungstic, hydroferrocyanic and metaphosphoric acids, precipitate proteins in acid solution only, while only few reagents are known, e.g. salts of heavy metals, which effect precipitation in neutral or weakly alkaline solutions. These reagents suffer from the disadvantage that they tend to denature the proteins. Simple salts, such as Na_2SO_4, work only in high concentrations.

It has now been found that complex salts can be used advantageously as protein precipitants, and from the numerous selection of complex salts available a variety of conditions are possible under which the precipitations can be carried out. Apart from the work of Bungenberg de Jong & Saubert [1936], the literature appears to contain no reference to the general use of complex salts as protein precipitants. These authors used complex salts in certain systems which produce what they term "coacervates", but the systems studied were of a more complicated nature than those in our investigations inasmuch as they comprised protein + complex salts + a third component which was usually an inorganic salt. With regard to the mechanism involved in such precipitations, the results of our quantitative experiments support the view that stoichiometric relations exist between protein and precipitant, as found by Chapman *et al.* [1927] for acid dyes, Meyer *et al.* [1937] for chondroitin sulphuric acid, and by Hermann & Perlmann [1937; 1938] for metaphosphoric acid. We have considered both the qualitative and quantitative aspects of the problem.

Qualitative experiments

Methods. The native protein was dissolved in dilute hydrochloric acid to give a 1% solution having *p*H 3. A freshly prepared aqueous solution of the complex salt was then added in such amount that the proportion by weight of protein to complex salt was 5 : 1. The appearance or non-appearance of a precipitate was then noted. An analogous procedure was then followed at the other *p*H values tabulated in Table I. Solutions having a *p*H value more

Table I

	Edestin at *p*H			Serum albumin at *p*H			Globin at *p*H		
Complex salt	3	4–6	7–9	3	4–6	7–9	3–7·5	8–9	9–11
$[Co(NH_3)_6]Cl_3$	−	+	+	−	+	+	−	+	+
$[Co(NH_3)_3(NO_2)_3]$	−	+	−	−	+	−	−	+	−
$K_3[Cr(C_2O_4)_3]$	+	+	−	+	+	−	+	+	−

+ = precipitate. − = no precipitate.

alkaline than the isoelectric point of the protein were obtained by adding $N/10$ NaOH until the desired pH was attained. Precipitation, when it did occur, was instantaneous and the precipitates were flocculent and settled easily. Table I shows that edestin and serum albumin are precipitated in acid solution by the anionic complex salt $K_3[Cr(C_2O_4)_3]$ (these proteins are normally insoluble within their isoelectric range, pH 4–6); in more alkaline solutions, precipitation occurs with the kationic complex salt $[Co(NH_3)_6]Cl_3$. On the other hand, a basic protein, globin, is precipitated in the whole range from pH 3 to 7 by the anionic complex salt $K_3[Cr(C_2O_4)_3]$. Within its isoelectric range, pH 8–9, globin is insoluble, while in more alkaline solutions it is precipitated by the kationic complex salt $[Co(NH_3)_6]Cl_3$. The complex salt $[Co(NH_3)_3(NO_2)_3]$, which is not ionized in aqueous solution, does not cause precipitation of any of the proteins studied, over the whole pH range. Precipitates of proteins with complex salts redissolve when the pH of the supernatant solution is altered; thus the precipitates formed by the addition of $K_3[Cr(C_2O_4)_3]$ to edestin and serum albumin were dissolved when the solutions were adjusted, by the addition of NaOH, to pH 7. The precipitates formed by the proteins with $[Co(NH_3)_6]Cl_3$ were readily soluble in dilute acid, and the precipitate of globin with $[Co(NH_3)_6]Cl_3$ was dissolved when the pH was adjusted to 7. Tests were carried out in the same way with a number of different complex salts. We quote as some typical representatives for the anionic complex salts:

$K_3[Cr(CNS)_6]$, $K[Cr(CNS)_4(NH_3)_2]$, (Reinecke salt), $K_3[Co(CN)_6]$,

and as typical representatives for kationic complex salts:

$[Cr(NH_3)_6]Cl_3$, $[Co(NH_3)_4CO_3]Cl$, $[Cr(en)_3]Cl_3$, $[Pt(SC(NH_2)_2)_4]Cl_2$.

It has been found that the precipitation of native proteins can be carried out with all ionized complex salts, provided that anionic complex salts are used at a pH which is more acid than the isoelectric point of the protein, and kationic complex salts are used at a pH which is more alkaline than the isoelectric point of the protein. With a proper choice of complex salts precipitation of proteins can, therefore, be effected over a wide pH range.

Quantitative experiments

An analytical study of some precipitates obtained with protein and complex salts was now made. Both types of complex salts, anionic and kationic, were used in this investigation, the precipitations being carried out under the general conditions outlined above.

Experimental procedure. The proteins under examination were dissolved in dilute acid or alkali and the solutions then adjusted to a known pH value. The complex salts which served as precipitants were dissolved and, when necessary, the pH value of the solution was adjusted to that of the protein solution. In each case the two solutions were mixed and the precipitate which formed was immediately centrifuged off, washed 4–5 times with water until the washings contained no protein or complex salt, washed twice with alcohol, then twice with ether, and finally dried in a vacuum over $CaCl_2$. The relative amounts of complex ion and protein in the precipitate were then estimated by analysis. The metallic content of the precipitate was determined and in one case the N contents of the precipitate and the original protein were compared. The experiments were confined to complex salts which contained chromium as central atom, because the method of Smith & Sullivan [1935] for chromium determinations in organic compounds offered a reliable and rapid method for

the estimation of the metal. The nitrogen determinations were carried out using Pregl's micro-Kjeldahl method.

Exp. 1. Examination of a typical precipitate from a protein and a complex salt. A crude preparation of insulin[1] (200 mg.) was precipitated in $N/20$ HCl (20 ml.) with a 5% solution of $K_3[Cr(C_2O_4)_3]$ (1·2 ml.).

Table II

Protein	% N content	% Cr content	% $[Cr(C_2O_4)_3]$ in ppt. calc. from Nitrogen	% $[Cr(C_2O_4)_3]$ in ppt. calc. from Chromium
Crude insulin	12·3	—	—	—
Insulin chromic oxalate	10·97	1·72	10·4	10·4

Table II shows that the amount of the complex anion $[Cr(C_2O_4)_3]$ in the precipitate (as estimated from the Cr content of the precipitate) is identical with the amount calculated from the difference in N content between the original protein and the precipitate. The Cr content of the precipitate may, in this instance, therefore, be taken as a true indication of the amount of complex ion present in the precipitate. A comparison between N and Cr contents could be carried out only in the case of chromic oxalate, because all other complex salts used in these experiments contained nitrogen.

Exp. 2. (*a*) *Precipitation of protein in acid solution with an anionic complex salt.* In this series both "crude" insulin and crystalline insulin were precipitated with varying amounts of the complex salt $K_3[Cr(CNS)_6]$. The precipitations were carried out either in $N/20$ HCl or in 2% acetic acid solution. The conditions were varied with respect to the relative amounts of protein and complex salt, to the concentrations of the protein and the complex salt and to the pH. The reactions were carried out in small centrifuge tubes, the final volume of the mixture always being adjusted to 10 ml.

Table III. *Precipitation of insulin with $K_3[Cr(CNS)_6]$ in acid solution*

Protein	Wt. of protein mg.	Wt. of $K_3[Cr(CNS)_6]$ mg.	% Cr in ppt.	% Cr in ppt. (average)	% $[Cr(CNS)_6]$ in ppt. calc. from % Cr
		(*a*) Precipitation in $N/20$ HCl			
Crude insulin	200	60	1·62		
,,	200	60	1·74		
,,	200	60	1·64	1·62	12·45
,,	200	60	1·55		
,,	200	60	1·57		
,,	200	80	1·69	1·69	13·0
,,	200	90	1·64	1·61	12·4
,,	200	90	1·57		
,,	200	100	1·62	1·62	12·45
,,	200	150	1·61	1·61	12·4
,,	150	60	1·60	1·60	12·3
,,	250	60	1·62	1·62	12·45
,,	300	60	1·54	1·54	11·9
Crystalline insulin	200	60	1·70	1·70	13·1
		(*b*) Precipitation in 2% acetic acid			
Crude insulin	250	75	1·57	1·57	12·1
,,	250	115	1·59	1·59	12·2

Table III shows that the amounts of complex ion $[Cr(CNS)_6]$ combined with the protein (as determined by the chromium content of the precipitates) were constant within the limits of experimental error (*ca.* 10%).

[1] A specimen of insulin which was not fully purified and not biologically standardized.

(*b*) *Precipitation of a protein with a kationic complex salt.* A series of experiments, analogous to those described above under (*a*), was carried out using the kationic complex salt $[Cr(CO(NH_2)_2)_6]Cl_3$. Casein was chosen as the protein since it is readily soluble at *p*H 7, and this *p*H is sufficiently removed from the isoelectric point of the protein. The casein was dissolved in dilute NaOH to give a 1 % solution having *p*H 7. An aqueous solution of the complex salt was added. The relative amounts of protein and complex salt were varied.

Table IV. *Precipitation of casein with* $[Cr(CO(NH_2)_2)_6]Cl_3$ *at* p*H 7*

Protein	Wt. of protein mg.	Wt. of $[Cr(CO(NH_2)_2)_6]Cl_3$ mg.	% Cr in ppt.	% Cr in ppt. (average)	% $[Cr(urea)_6]$ in ppt.
Casein	200	40	1·05	1·05	8·35
,,	200	60	1·07	1·07	8·5
,,	200	60	1·07		
,,	250	25	0·93	0·95	7·55
,,	250	25	0·87		
,,	250	25	1·06		
,,	250	80	1·00	1·00	7·95
,,	250	100	1·06	1·08	8·65
,,	250	100	1·11		

Table IV shows that whereas the weight of complex salt added was varied by as much as 400%, the maximum variation in the amount of complex ion in the precipitate was only 12%. It appears from the results shown in Tables III and IV that a given protein, when precipitated with a complex salt, combines with it in stoichiometric proportions, which are independent of concentration and relative amounts of protein and complex salt present.

Exp. 3. Determination of equivalent of complex ion combined with protein in the precipitate. (*a*) *Anionic complex ions.* In the experiments recorded in Table V each of the proteins was precipitated with three different complex salts, two of which had trivalent complex anions, while the third (Reinecke salt) had a monovalent complex anion. The figures in column 3 show the percentage of complex ion in the precipitates. It will be seen that for a given protein these values vary considerably for different precipitants. When, however, these figures are recalculated with reference to the equivalent weights of the complex ions and expressed for 1 g. protein (shown in column 4) it will be seen that the

Table V. *Precipitations with anionic complex salts in acid solution*

1 Protein	2 Complex ion*	3 % Complex ion in ppt.	4 M.-equiv. of complex ion combined with 1 g. protein	5 Average
Crude insulin	$[Cr(CNS)_6]'''$	12·4	1·06	—
,,	$[Cr(C_2O_4)_3]'''$	10·6	1·13	1·04
,,	$[Cr(CNS)_4(NH_3)_2]'$	22·6	0·92	—
Crystalline insulin	$[Cr(CNS)_6]'''$	13·1	1·12	1·12
Egg albumin	$[Cr(CNS)_6]'''$	9·4	0·78	—
,,	$[Cr(C_2O_4)_3]'''$	7·0	0·715	0·75
,,	$[Cr(CNS)_4(NH_3)_2]'$	19·4	0·76	—
Clupein	$[Cr(CNS)_6]'''$	34·1	3·90	—
,,	$[Cr(C_2O_4)_3]'''$	30·4	4·15	4·1
,,	$[Cr(CNS)_4(NH_3)_2]'$	58·0	4·35	—

* The salts used were the potassium salts.

equivalents of the complex ions combined with a given protein are essentially the same (within the limits of experimental error), and moreover are independent of the nature and the valency of the complex ion used. It is also evident from these figures that the trivalent complexes combine with the proteins as "tribasic acids", while the monovalent complex combines as a "monobasic acid".

(*b*) *Kationic complex ions.* In the experiments recorded in Table VI various proteins were precipitated in approx. neutral solution with kationic complex salts. The two complex salts used have trivalent complex kations, but their

Table VI. *Precipitations with kationic complex salts at* p*H 7–8*

1 Protein	2 Complex ion*	3 % Complex ion in ppt.	4 Complex combined m.-equiv./g. protein	5 Average
Casein	$[Cr(urea)_6]^{+++}$	8·30	0·66	0·68
,,	$[Cr(NH_3)_6]^{+++}$	3·52	0·71	
Amorphous insulin	$[Cr(urea)_6]^{+++}$	5·3	0·41	0·415
,,	$[Cr(NH_3)_6]^{+++}$	2·15	0·42	
Crystalline insulin	$[Cr(urea)_6]^{+++}$	4·8	0·37	0·37
Egg albumin	$[Cr(NH_3)_6]^{+++}$	1·95	0·39	0·39

* The salts used were the chlorides.

molecular weights (412 and 154 respectively) are far apart. Thus the combining weights of these complex ions differ considerably, and this difference is significant, inasmuch as it excludes any accidental agreement of results. The values found for the two complex ions combined with a given protein are in fairly good agreement. Thus casein combined with 0·66 and 0·71 m.-equiv./g. of protein in the case of $[Cr(urea)_6]Cl_3$ and $[Cr(NH_3)_6]Cl_3$ respectively. The agreement between the values of the two complex ions is even better in the case of amorphous insulin (0·41 and 0·42 m.-equiv./g. of protein respectively). The corresponding figure for crystalline insulin is slightly lower (0·37). These results show that a protein combines on the alkaline side of its isoelectric point with a definite equivalent of complex kations, independently of the nature of the complex salt used.

The precipitation of proteins with complex salts appears, therefore, to take place in such a manner that each protein displays a definite capacity for combining with acid or basic complex ions.

The averaged values of acid- and base-binding capacities shown in column 5 of Tables V and VI are compared in Table VII with values which have been found by other methods.

The values for insulin found by precipitation with complex anions are slightly higher than the values obtained by Harington & Neuberger [1936] by means of electrometric titration. The figure (0·75), calculated from our experiments for egg albumin, is higher than the value (0·61) which Meyer *et al.* [1937] found from precipitation experiments with chondroitin sulphuric acid, but is in good agreement with the figure (0·78) obtained by Perlmann & Herrmann [1938] from precipitation experiments with metaphosphoric acid. There is good agreement between our value for clupein and that of Felix & Mager [1937] calculated from the combination of clupein with various acids.

Our values for the base-combining capacities of proteins are of the same order as those found by other methods in the case of insulin and casein. Our value for egg albumin, on the other hand, is much smaller than the value of the "maximal base-combining capacity" given by Cohn *et al.* [1925].

Table VII. *Acid- and base-combining capacities of some proteins determined by different methods*

Protein	Acid-combining capacities m.-equiv./g. protein: Complex ppt.	Other methods	Method	Author
Amorphous insulin	1·04	—	—	—
Crystalline insulin	1·12	1·01	Electrometric titration	Harington & Neuberger
Egg albumin	0·75	0·61	Precipitated with chrondroitin sulphuric acid	Meyer *et al.*
	—	0·78	Precipitated with HPO_3	Perlmann & Hermann
Clupein	4·1	4·1	Combination with acids	Felix & Mager

Protein	Base-combining capacities m.-equiv./g. protein: Complex ppt.	Other methods	Method	Author
Amorphous insulin	0·41	0·45	Electrometric titration	Harington & Neuberger
Crystalline insulin	0·37	—	—	—
Casein	0·68	0·55	Ca-salts	Van Slyke [1915]
Egg albumin	0·39	0·8	Maximal base-combining capacity, electrometric titration	Cohn *et al.*

Discussion

Attention is drawn to the fact that all ionized complex salts possess the capacity of precipitating soluble proteins from their solutions. Salts which possess a complex anion precipitate proteins only when the latter are in kationic state, i.e. at a *p*H more acid than the isoelectric points of the proteins; salts which possess complex kations precipitate proteins only when the protein is present in its anionic state, i.e. at a *p*H more alkaline than the isoelectric point of the protein. This rule applies to all complex salts and all native proteins which have been examined. The precipitation appears to be, therefore, the effect of a salt-like combination of the complex ion with the protein carrying the opposite charge.

We can conclude that the complex ions remain intact in the precipitates from the fact that precipitation is instantaneous and that the colour of the complex ion is retained in the precipitate: e.g. protein precipitates with Reinecke salt are red, with $[Cr(urea)_6]Cl_3$ green, with $[Cr(NH_3)_6]Cl_3$ orange-yellow. The precipitations described here are, therefore, different in character from those which Elöd & Schachousky [1935] observed when gelatin was heated with certain unstable complex salts.

The results of our analytical determinations indicate that proteins, when precipitated with a complex salt, combine in stoichiometric proportions with the complex kation or anion respectively. This observation finds a parallel in the combination of certain amino-acids with complex salts [Bergmann, 1935], e.g. *l*-proline rhodanilate, $[Cr(CNS)_4(C_6H_5NH_2)_2].(C_5H_{10}O_2N)$, in which case the monovalent complex anion is combined with the monovalent amino-acid, the proline reacting in its kationic form.

We can conclude from our figures that proteins combine in acid solution with the complex anion with their "maximal acid-combining capacity", i.e. that all basic groups of the protein are able to react with the precipitant. This is shown in the case of insulin by the agreement of our figure with the highest value of acid-combining capacity found by electrometric titration; it is also in agreement

with Chapman's observation that acid dyes combine with proteins at *p*H 2–2·5 in constant proportions and in amounts which are to be expected when all basic groups are ionized. Our figures for the base-combining capacities of proteins indicate, on the other hand, that the proteins, when precipitated with kationic complex salts at or near *p*H 7, do not combine with their "maximal base-combining capacity". A possible explanation is that the phenolic hydroxyl groups are not ionized and therefore do not react.

That precipitation of proteins with complex salts is not accompanied by coagulation or denaturation of the protein is shown by the ease with which the precipitates are redissolved on appropriate change of *p*H, and by the fact that the precipitations are often reversed by certain neutral salts, such as acetates, phosphates and ammonium salts.

The degree of precipitation of a given protein varies considerably with different complex salts, but we have not detected any considerable differences in the degree of precipitation of different proteins by one and the same complex salt.

Summary

1. It has been shown that ionized complex salts precipitate proteins from their aqueous solutions.

2. Anionic complex salts precipitate proteins at a *p*H more acid than the isoelectric point of the protein, whereas kationic complex salts precipitate at a *p*H more alkaline than the isoelectric point.

3. These precipitations are not accompanied by denaturation or coagulation of the protein.

4. Quantitative estimations of the N and metal contents of such precipitates have shown that the proteins combine with a definite equivalent of complex ion, independently of the relative proportions and concentrations of the reagents, i.e. in stoichiometric proportions.

5. The amounts of complex ions combined in the precipitates with various proteins represent, with some reservations, the acid- and base-combining capacities of the proteins; they were in several cases found to be in reasonable agreement with values calculated from other determinations.

6. It appears that proteins, when precipitated in acid solution with complex anions, combine with their maximal acid-combining capacity, whereas proteins, when precipitated with complex kations in approximately neutral solution, combine with only a fraction of their maximal base-combining capacity.

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CXIV. THE ISOLATION OF 17-KETOSTEROIDS FROM THE URINE OF NORMAL WOMEN

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In a recent paper [Callow, 1939] a chromatographic method of separating the ketones in the neutral fraction of an extract of men's urine was described. By this method androsterone, aetiocholan-3(α)-ol-17-one and *trans*dehydroandrosterone could readily be isolated in greater yields than had previously been possible, and actually in amounts probably not very different from those originally present in the crude extract. The present paper describes the application of the same process to an extract of normal women's urine with the object of making a roughly quantitative comparison.

Experimental

Collection and extraction of urine. Combined extracts from a large number of experimental extractions of bulked normal women's urine, and from the urines of a few normal patients, were used. Most of the bulk collections were made in the Nurses' Home of the Middlesex Hospital. Only extracts of fresh urine, with *p*H from 5·5 to 7 before hydrolysis, were included. The urine was either hydrolysed by heating with HCl and then extracted with benzene, or a combined hydrolysis and extraction was carried out, as in the method of Dingemanse *et al.* [1937; cf. Callow *et al.* 1939]. The extracts were separated into neutral, acidic and phenolic fractions. After colorimetric and capon assays had been carried out, alcoholic solutions of the neutral fractions were combined and the alcohol was removed. The residue from 114 l. of urine was taken up in 200 ml. of methanol, left overnight at 0° and filtered. Evaporation of the methanol gave 3·6 g. of residue. Treatment with the Girard-Sandulesco reagent P gave 1·14 g. of ketonic fraction, i.e. 10 mg./l. of original urine, and 0·91 g. of non-ketonic fraction. Colorimetry indicated 55 % of 17-ketosteroids in the ketonic fraction [cf. Callow *et al.* 1938] and a capon assay, for which we are indebted to Mr C. W. Emmens, an androgenic activity of 1·45 I.U./mg., or 14·5 I.U./l. of original urine. The non-ketonic fraction contained 0·25 I.U./l. Half of this brown, gummy ketonic fraction, equivalent to 57 l. of original urine, was taken up in 25 ml. of carbon tetrachloride and put through a 27 × 1·5 cm. column of alumina as previously described. After development with about 800 ml. of carbon tetrachloride, it showed the following coloured bands, reading from top to bottom—brown, red, white, faint pink, faint yellow, white, yellow. Successive portions of 650–800 ml. of the eluate were evaporated, yielding fractions I, II and III, wt. 26, 16 and 6·5 mg. respectively. Fraction I was a gum (72 % ketone); Fraction II crystallized partially on standing, but the crystalline material has not yet been identified; Fraction III included the lowest yellow band. Evaporation of a further portion of about 700 ml. of carbon tetrachloride eluate gave only a trace of gummy residue, which was discarded. A total of 2900 ml. of carbon tetrachloride had been used at this stage. The column was then eluted with a total of 1900 ml. of carbon tetrachloride containing 0·1 % of alcohol. Evaporation of successive

portions of eluate gave 7 fractions (IV–X), details of which are given in Table I. As with the extract of men's urine, this treatment caused a sharply defined yellow band to appear and to be washed rapidly down the column (Fraction V). Fractions VII, VIII, IX and X were crystalline.

Table I

Fraction	Vol. of eluate ml.	Solvent	Wt. of fraction mg.	M.P. (crude fractions) °C.	$[\alpha]_D$(EtOH)	$[\alpha]_{5461}$(EtOH)	Remarks
—	230	0·1% EtOH in CCl_4	Trace	—	—	—	Discarded
IV	200	,,	10	—	—	—	Gum
V	90	,,	16	—	—	—	Yellow band
VI	220	,,	29	—	+ 45·7°	+ 58·7°	Gum: 8 mg. *trans*dehydroandrosterone isolated
VII	200	,,	14	126–161	+ 85°	+110°	Chiefly androsterone
VIII	280	,,	35	166–178	+ 96°	+120°	Androsterone
IX	300	,,	24	156–180	+ 92°	+108°	,,
X	380	,,	5·7	140–178	—	—	,,
XI	300	0·2% EtOH in CCl_4	Trace	—	—	—	
XII	300	,,	29	—	+ 80°	+100°	Gum
XIII	250	,,	24·5	125–144 Tr. 140	+101°	+122°	Aetiocholan-3(α)-ol-17-one
XIV	180	,,	15	130–150 Tr. 135	+100°	+120°	,,
XV	600	,,	36	110–146	+ 96°	+110°	Aetiocholan-3(α)-ol-17-one +red colouring matter
XVI	430	,,	5·7	—	—	—	Gum

Elution was continued with carbon tetrachloride containing 0·2% of alcohol. The first 1630 ml. of eluate were divided into five fractions (XI–XV, Table I), the last of which included the red colouring matter. Further elution (XVI and subsequent fractions) gave small quantities of gummy material which were not examined further.

Isolation of steroids from crude fractions. (*a*) trans*Dehydroandrosterone.* Fractions IV and V were not further examined. From the behaviour of the extract of men's urine it was suspected that *trans*dehydroandrosterone would be found in Fraction VI, which was a yellow gum, weighing 29 mg. Its specific rotation was comparatively low, and colorimetry indicated 67% of 17-ketosteroids. The fraction was treated with 150 mg. of digitonin in 14 ml. of 60% aqueous alcohol. The precipitate which formed overnight was collected and washed with a little ether. It weighed 49 mg. It was then decomposed with pyridine, the digitonin precipitated by the addition of ether, and the solution filtered, washed with dilute HCl and water, dried and the ether removed. The crystalline residue weighed 8·5 mg., and had M.P. 127–140° and $[\alpha]_D$ +18° (EtOH). The identity of the compound with *trans*dehydroandrosterone was established by preparation of the benzoate. This was very sparingly soluble in methanol; recrystallized from ethyl acetate, it had M.P. 243–252°, and a mixture with an authentic specimen of *trans*dehydroandrosterone benzoate had M.P. 250–254·5° (soft at 243·5°).

The melting point and specific rotation suggested that Fraction VII might be a mixture of androsterone and *trans*dehydroandrosterone. An approximate absorption spectrum of the colour produced with Zimmermann's reaction showed that it was about 90% 17-ketosteroid. It was taken up in 1·5 ml. of 50% aqueous alcohol and treated with a hot solution of 80 mg. of digitonin in 8·5 ml. of 50% alcohol. After standing overnight the precipitate was collected and weighed. Yield: 7·5 mg. This was decomposed in the usual way and the product benzoylated, when the characteristic benzoate of *trans*dehydroandrosterone was obtained. The total yield of *trans*dehydroandrosterone from Fractions VI and VII was about 10 mg.

(*b*) *Androsterone.* The filtrate from the above digitonin precipitate was extracted with ether, the extract washed with water, dried and the ether removed. The slightly gummy crystalline residue weighed 12 mg. Recrystallized from methanol it melted at 178–181·5°, after subliming on to the cover slip. It was combined with later androsterone fractions for further recrystallization. The specific rotations and melting points of the crude Fractions VIII and IX suggested that they were androsterone. An approximate absorption spectrum of the colour reaction was that of a 17-ketosteroid. Fraction VIII recrystallized from methanol gave plates, M.P. 181–183°, after subliming to needles, and, mixed with an authentic specimen of androsterone, melted at 179–183°. The identity of the compound as androsterone was confirmed by preparation of the oxime. This, recrystallized from aqueous alcohol, had M.P. 208–211°, which was unchanged by admixture with an authentic specimen. Fraction IX recrystallized from methanol gave 10 mg. of plates M.P. 182–184° after subliming to needles. Mixed with an authentic specimen of androsterone, the M.P. was 183–185°. The total amount of androsterone isolated in a moderately pure state was 76 mg. It may be noted that this quantity accounts for 90% of the biological activity of the crude ketonic fraction as measured by the comb-growth method.

(*c*) *Aetiocholan-3(α)-ol-17-one.* The crystalline Fractions XIII, XIV and XV were separately treated with charcoal in methanol solution. Addition of water to the filtrates gave material crystallizing in long needles which partially melted and changed to a platy crystalline form at 140–143° and finally melted at about 150°. This behaviour suggested that the material in all cases was aetiocholan-3(α)-ol-17-one, which has a transition point at 140–142°, but a sharp M.P. could not be obtained. The fractions were therefore combined and converted into the benzoate. This had M.P. 161·5–163·5° (from methanol) and the M.P. was unchanged by admixture with an authentic specimen of aetiocholan-3(α)-ol-17-one benzoate. About 76 mg. of aetiocholan-3(α)-ol-17-one were isolated in a moderately pure state.

(*d*) *Unidentified material.* In addition to the small amount of crystalline material separating from Fraction II already mentioned, there is an indication of an unidentified material in the non-crystalline Fraction XII. The position in the series, the specific rotation (see Table I) and the 17-ketosteroid content (72%) suggest that it might be a mixture of aetiocholan-3(α)-ol-17-one with a non-ketonic impurity, but the presence of other compounds cannot be excluded. A crystalline benzoate, M.P. 80–140°, was prepared, but the amount was not sufficient for purification. Further investigation of these materials will require larger amounts of urine extract.

Note. All melting points recorded were observed in Kofler's micro-melting point apparatus. The optical rotations were measured in a 4 dm. tube.

Discussion

The origin of the 17-ketosteroid compounds in women's urine is as yet a matter only for surmise. Androsterone and aetiocholan-3(α)-ol-17-one were isolated in abnormally high yield from the urine of a man receiving testosterone [Callow, 1939]. This observation indicates that testosterone is transformed by the body into androsterone and aetiocholan-3(α)-ol-17-one. The further observations that these two compounds were major constituents of the neutral ketonic fraction of extracts of normal men's urine was consistent with the assumption that they were derived from endogenous testosterone, assumed to be secreted by the testis. The observations now reported show that normal women's urine yields 1·3 mg./l. of androsterone and 1·3 mg./l. of aetiocholan-3(α)-ol-17-one, as

compared with the yields of 1·6 mg./l. and 1·4 mg./l., respectively, now obtained from normal men's urine by the same method. The excretion of these two compounds is, therefore, not an index of the secretory activity of the testis. The simple assumption that these compounds are excretory transformation products characteristic of testosterone only, would lead to the conclusion that testosterone was secreted by the normal woman in amounts comparable with those secreted by the normal man.

There are, however, no grounds for the assumption that these compounds cannot also be derived from other steroids in the body, whether of gonadal or of adrenal cortical origin. Although ovaries may secrete material with androgenic activity [Hill & Gardner, 1936; Parkes, 1937; Hill & Strong, 1938; Deanesly, 1938], the nature of the compounds produced is inknown. The idea that normal males and females both secrete the same highly active androgen in comparable amounts is contrary to current physiological theories, although Korenchevsky [1939] considers it reasonable to suppose that male hormones present in the female should be of the same nature as the other natural male hormones. The next step we propose to take in the chemical investigation is to examine the nature of the compounds excreted by eunuchs and ovariectomized women. Presumably this will give a clue to the contribution of the adrenal cortex to the urinary 17-ketosteroids. It may be that the latter will include the compounds now regarded as derived from testosterone. Up to the present the evidence points to *trans*dehydroandrosterone being a characteristic transformation product from the adrenal cortex in tumour cases [Callow, 1936; Crooke & Callow, 1939], whilst Butler & Marrian [1938, 1, 2] have isolated aetiocholan-3(α)-ol-17-one and androstan-3(β)-ol-17-one from the urine of a case of adrenal hyperplasia. It may be noted that the amounts of *trans*dehydroandrosterone now isolated from urine of normal women, although higher than those obtained previously [Callow & Callow, 1938], are still small and comparable with those from normal men's urine.

Summary

1. Aetiocholan-3(α)-ol-17-one (1·3 mg./l.), androsterone (1·3 mg./l.) and *trans*dehydroandrosterone (0·2 mg./l.) have been isolated from extracts of the urine of normal women. These yields are comparable with those of the same compounds from normal men's urine.

2. Androsterone is the principal androgenic compound in extracts from the urine of either sex.

3. Although urinary aetiocholan-3(α)-ol-17-one and androsterone are known to be derived from testosterone when this has been given by injection, it does not follow that their presence in urine indicates the secretion of testosterone by women.

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CXV. GROWTH AND REPRODUCTION ON A LOW FAT DIET

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The low fat diet employed by the Burrs [Burr & Burr, 1929; 1930; Burr *et al.* 1932] in their work on the essential fatty acid has served as a model in the search for new fat-soluble dietary essentials. The diets used in such investigations have, without sufficient evidence, been generally referred to as "fat-free", "rigorously fat-free" etc. Failures to disclose the existence of new factors of a lipid nature are qualified, therefore, by the absence of quantitative data on the fat contents of these diets. In addition, there exist in the literature suggestions, both implied in the results reported and specifically stated, that fat-soluble factors other than vitamins A, D and E, and the essential fatty acid, are required by the rat [Burr & Burr, 1929; Mason, 1929; Coward *et al.* 1929; Guha, 1931; Mapson, 1932; Blumberg, 1935; Marchesi, 1935; Olcott & Mattill, 1937; Martin, 1939].

When analysis disclosed that several components of so-called "fat-free" diets contained appreciable amounts of lipid, the preparation of a diet lower in lipids than any previously fed was successfully attempted. The analytical findings, the preparation of the diet and the results of the subsequent feeding experiments are reported in this paper.

Analysis of dietary components and preparation of a low fat diet

"Fat-free" diets have generally been composed of a salt mixture, sucrose, casein (ether- or alcohol-extracted or both), ether-extracted yeast and pure or concentrated sources of vitamins A (or carotene), D and E, and the essential fatty acid. Since the degree of purity of the fat-soluble essentials used in these diets is generally apparent, and since methods for purifying them are available, attention was first turned to the lipid contents of extracted yeast and casein.

Analysis of dietary components. The Liebermann saponification method for fat determination as described by Leathes & Raper [1925] was found most suitable for the analysis of yeast. By acidifying and extracting the entire saponified mixture, a measure of all light petroleum-soluble substances was obtained.

Table I. *Lipid content of dried yeast before and after ether extraction*

	Lipid content %		
Brand of yeast	Unextracted	Ether-extracted 24 hr.	Ether-extracted 48 hr.
Northwestern Yeast Foam Tablet Powder	4·2	3·7	3·7
Anheuser-Busch Strain C	5·7	5·5	5·5
Fleischmann No. 1550	5·8	4·6	4·5

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Three brands of dried yeast, unextracted and extracted for periods of 24 and 48 hr. in the Soxhlet apparatus with C.P. anhydrous ethyl ether, were examined. The average figures (Table I) show differences in the yeasts both with respect to total lipids and the % lipids removed by ether extraction; but in each case it is clear that most of the fatty substances could not be removed by this method. Whether this was due to their inaccessibility to the solvent, or to their existence in insoluble chemical combinations was not determined.

In order to eliminate the possibility of the conversion of sugars or amino-acids into light petroleum-soluble substances during the strong alkali hydrolysis, control experiments were made with sucrose and casein but with negative results. Furthermore, ether-extracted yeast cells stained for fat with Sudan III gave positive results, as evidenced by the appearance of many bright red globules. Thus the assumption that ether-extracted yeast furnishes a "fat-free" source of the vitamin B complex was shown to be erroneous.

Two methods, the Liebermann saponification [Leathes & Raper, 1925] and the Shaw [1920] adaptation of the Roese-Gottlieb procedure, were investigated as to their accuracy in determining the lipid content of casein. Since the Liebermann method gave much lower values owing to the formation of a fat-including coagulum, the method of Shaw was accepted as superior in this case. The results of analyses made on samples of casein subjected to extraction with hot or cold alcohol and ether, or to successive extractions with these solvents, are given in Table II. Ether extraction of crude casein is much less

Table II. *Lipid content of extracted casein*

Casein	Treatment	Lipid content %
Crude	None	1·45
Crude	None*	1·50
E.E.	Ether-extracted*	1·48
CA	Cold alcohol-extracted†	0·24
CA 1	Cold alcohol + 24 hr. warm ether extraction‡	0·17
CA 2	Cold alcohol + 48 hr. warm ether extraction	0·13
CA 3	Cold alcohol + 72 hr. warm ether extraction	0·12
CA 4	Cold alcohol + 24 hr. hot 95 % alcohol extraction	0·025
CA 5	Cold alcohol + 24 hr. warm ether and 24 hr. hot 95 % alcohol extraction	0·02

* Figures taken from Shaw [1920].

† Acetic acid-washed and continuously extracted in a Lloyd extractor with cold 70 % and cold 95 % alcohol for 24 hr. each.

‡ This and all subsequent extractions carried out in Soxhlets.

efficient than cold 70 and 95 % alcohol extraction, which removes approximately 83 % of the lipids. Furthermore, it is seen that the removal of lipid from this cold alcohol-extracted casein is more readily accomplished by continuous extraction with hot 95 % alcohol than with warm anhydrous ethyl ether. The successive application of these two last-named solvents does not seem to reduce the lipids to a significantly lower level than extraction with hot 95 % alcohol

alone. Indeed, in both of these cases the results of the analysis are in the range of the blank determinations, but since there generally seemed to be one or two droplets of lipid in the weighing vessels no corrections were made. It would appear from Table II that many "fat-free" diets have contained milk fat in addition to yeast fat.

Since no lipid was found in commercial sucrose analysed by the Liebermann method, the sucrose used in "fat-free" diets has probably not been a source of contamination.

Of the known fat-soluble essentials used in these diets, provitamin A and vitamin D have recently been included in pure forms; vitamin E has, with a few exceptions, been added as the crude unsaponifiable matter of wheat germ oil, and the unsaturated fatty acid has been supplied (in long term experiments) by a distillate containing a mixture of esters.

Preparation of a low fat diet. (Low fat diet is used throughout the remainder of this paper to refer to a diet whose lipid content is small.) Following unsuccessful attempts to remove lipids completely from yeast, a satisfactory source of the vitamin B complex was extracted in the following manner. 100 g. Northwestern Yeast Powder were added to 1 l. 50 % alcohol and the mixture was rapidly stirred for 2 hr. The mixture was allowed to stand for 1 hr. and the extract was then filtered through No. 52 Whatman filter paper. Most of the alcohol was removed by concentrating the filtrate on the water pump at 40–50° to a volume of about 200 ml. The concentrate was shaken for 3 min. in a separating funnel with an equal volume of C.P. anhydrous ethyl ether. This extraction was repeated. Liebermann analysis showed the fat content of the resulting defatted yeast extract in terms of whole yeast to be 0·01 %.

The extract was assayed for growth, reproduction and lactation on vitamin B complex-deficient rats. The extract from 100 g. of yeast added to 900 g. of diet produced excellent growth in depleted male rats, an average gain of 300 g. over a period of 17 weeks. Female animals that had gained 120 g. in 6·5 weeks and were then mated produced and weaned good litters. The results obtained with the extract at this level were comparable in every respect with those obtained on 10 % whole yeast.

The preparation of a low fat casein is indicated in Table II (Casein CA5). While ether extraction in addition to hot alcohol extraction had no apparent effect on the lipid content, this step was included as an added precaution. The hot 95 % alcohol extraction was carried out in all-glass Soxhlets.

Methyl linoleate was prepared from cottonseed oil by the method of Rollett [1909]. This procedure was facilitated by substituting a 1 : 1 light petroleum (B.P. 30–60°): carbon tetrachloride mixture for ligroin in recrystallizing the tetrabromostearic acid. The purity of the preparation was attested by the melting-point of the tetrabromide, 114–115°, the figure obtained by Rollett, and the iodine number of the ester, 172·3, the theoretical value being 172·2.

A method for preparing a highly potent concentrate of vitamin E for long-term feeding experiments where the use of the pure vitamin is not yet feasible has been reported [Mackenzie *et al.* 1938]. Although this concentrate had proved potent in single 3 mg. doses in rats reared on a typical lard-containing vitamin E-deficient diet, there was no assurance that the same level would suffice on a low fat diet. It was assayed at a 5 mg. level on female rats maintained on the low fat diet (described below) without vitamin E and found to be effective.

As the remaining components necessary for the completion of the diet, sucrose, cystine (as a supplement to the casein), salts, calciferol and carotene, presented no problems, the following diet was assembled.

Sucrose	63·85
Casein (extracted with cold 70 % and 95 % alcohol, warm ether and hot 95 % alcohol)	20·00
Cystine	0·05
Salt mixture, McCollum 51* + 1·6 % $MnSO_4$, $2H_2O$ ...	6·10
Yeast extract† equivalent to 10 g. yeast	
Carotene, 20 mg. per 900 g. diet	
Calciferol,‡ 0·2 mg. per 900 g. diet	

* $CaCO_3$ 1·5, KCl 1·0, NaCl 0·5, $NaHCO_3$ 0·7, MgO 0·2, KH_2PO_4 1·7, Fe citrate 0·5.

† Extract prepared from Northwestern Yeast Foam Tablet Powder.

‡ We are indebted to Dr C. E. Bills of Mead Johnson & Company, Evansville, Indiana, for the calciferol.

Supplements

Methyl linoleate, 25 mg. per rat 6 times weekly.
Vitamin E concentrate, 5 mg. per rat twice weekly.

Mixing the diet and care of animals. The yeast extract was poured on the casein and evaporated at room temperature before an electric fan. The carotene and calciferol were dissolved in C.P. anhydrous ethyl ether and immediately evaporated on separate portions of the casein-yeast extract mixture. The various components of the diet were then thoroughly mixed. The diet was prepared fresh every 3–5 days and stored in the ice box between feedings.

All-metal individual cages with raised mesh bottoms were used to house the animals. Food was supplied *ad libitum*, the supply being replenished daily, except once each week when the animals were allowed to consume their ration entirely, thus ensuring the complete intake of all components. Supplements were administered in the morning on a small amount of the basal diet and were always consumed by evening.

The maximum lipid content of the basal diet is 0·0078 %, of which 0·0022 is due to carotene and calciferol and 0·0056 to impurities in the casein and yeast extract. Of the two supplements, the methyl linoleate is pure, while the vitamin E concentrate contains at the most 70 % of extraneous matter.

The daily consumption of lipid, based on the average food intake of 10 g. per rat per day, is approximately 0·027 g., of which 0·025 g. is methyl linoleate. Thus the lipid content of the complete diet (basal plus supplements) may be given as 0·27 %. Similarly, the maximum non-vitamin lipid content of the complete diet is 0·0156 %, and of the diet without vitamin E, 0·0056 %.

Feeding experiments on the low fat diet

Animals obtained from the laboratory stock colony[1] were placed on the experimental diet when they were about 21 days old and weighed 35 g. The composite growth curves of one group of 10 male and one group of 10 female rats maintained on the diet for a period of 44 weeks are given in Fig. 1. The curves for the low fat and the stock females show an insignificant superiority in weight on the part of the low fat animals. The curve for males on the low fat diet diverges from the stock male curve at 12 weeks and is about 30 g. below at 44 weeks. The significance of this difference is questionable in view of the great individual variations within both groups.

The average time of cessation of growth (defined as a failure to gain more than 5 g. during the ensuing 6 weeks) for the female rats was 22 weeks. Some females maintained a constant weight until the experiment was terminated, while others after remaining at a constant weight for 7–12 weeks gained as much

[1] The McCollum stock diet is composed of wheat (soft) 20·0, maize (yellow) 20·0, rolled oats 20·0, flaxseed oil meal 10·0, casein (crude) 3·5, whole milk powder 25·0, $CaCO_3$ 0·5, NaCl 1·0, Fe citrate 0·0011, $CuSO_4$, $5H_2O$ 0·0004.

as 15 g. Eight male rats ceased to grow after an average of 32 weeks, while 4 were still growing at 40 weeks. Of the 8 males, 3 subsequently gained 15–35 g. and 5 held a constant weight for their remaining 8 weeks on the experiment. The erratic behaviour of the animals with respect to the time and duration of constant weight maintenance could not be correlated with different batches of diet.

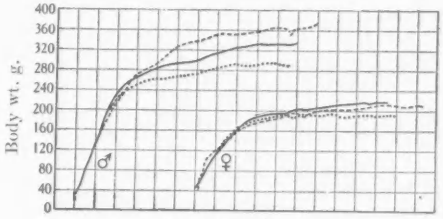

Fig. 1. Average growth curves of male and female rats receiving the low fat diet ———, the low fat vitamin E-deficient diet ....., and the stock diet - - - - - -.

The adequacy of the diet for growth was further tested on 4 groups of rats by doubling the intake of all vitamins and cystine, by increasing the vitamin E concentrate four-fold, by giving supplements of fresh calves' brain, and by replacing the low fat diet by the stock diet. With the exception of the experiments in which the vitamin and the cystine intakes were doubled, these changes were made with rats that had maintained constant weights for 6 weeks or longer. The growths of the control and test animals had been similar prior to the changes in diet. The results of these changes, summarized in Table III, were in all cases negative.

Table III. *Growth response as a result of supplementing or replacing the low fat diet for 4- and 8-week periods*

Change in diet	No. of rats	Sex	Av. time of change	Av. time of cessation of growth	Av. wt. at cessation of growth	Av. wt. at change	Av. wt. increase over wt. at cessation of growth	
							4 weeks	8 weeks
			weeks	weeks	g.	g.	g.	g.
Doubling all vitamins and cystine	3	♂	19	—	—	261	12	—
Controls on low fat diet	3	♂	20	—	—	260	12	—
Doubling all vitamins and cystine	2	♀	21	—	—	162	4	—
Controls on low fat diet	2	♀	20	—	—	182	6	—
Vitamin E concentrate, 40 mg. per week	3	♀	25	18	213	212	2	—
Controls on low fat diet	3	♀	31	25	191	191	4	—
Fresh brain, 1 g. daily	5	♀	28	21	199	196	2	6
Controls on low fat diet	5	♀	28	22	205	204	3	5
Stock diet	2	♂	36	30	279	278	10	14
Controls on low fat diet	4	♂	38	30	304	302	10	10
Stock diet	6	♀	37	30	196	193	1	1
Controls on low fat diet	6	♀	36	27	204	201	1	1

Since brain is an exceptionally rich source of lipids, 4 male and 4 female rats were placed at weaning on the low fat diet with the addition of 1 g. of fresh brain per rat daily and maintained on this regimen for 30 weeks. Their growth did not significantly differ from that of animals on the low fat diet.

The fertility of females on the low fat diet was demonstrated by the delivery of 14 litters by 5 rats mated to stock males. Following the second and third parturitions at 15 and 21 weeks, the behaviour of the mothers and the young was observed. Several days before the termination of pregnancy the mesh bottoms were removed from the cages and the females were placed on a bed of filter paper clippings previously extracted with hot 95 % alcohol. In all cases the females built nests and showed solicitude for their young, as was manifested by retrieving and hovering. Though the young were normal in appearance, many died during the first 3 days. Doubling the vitamin and cystine intakes on the 13th day of the 3rd pregnancy with 3 of the rats did not substantially improve the results. When young were living at 21 days they were, with the exception of those from one litter, poor in weight and appearance. The details of this experiment are summarized in Table IV.

Table IV. *Reproductive behaviour of females grown and maintained on the low fat diet*

	First gestation*		Second gestation				Third gestation			
Rat no.	No. of living young	Av. wt. at birth g.	No. of living young	Av. wt. at birth g.	Fate of young	Av. wt. at 21 days g.	No. of living young	Av. wt. at birth g.	Fate of young	Av. wt. at 21 days g.
C 5	9	4·1	7†	4·7	Dead 1 day	—	5‡	5·8	5 weaned	31·0
C 8	8	5·2	9	5·2	Dead 8 days	—	7‡	5·6	2 weaned	20·5
C 18	7	4·1	8	4·5	Dead 2 days	—	10‡	4·1	Dead 3 days	—
C 24	6	4·7	10	4·6	Dead 1 day	—	11	4·8	1 weaned	19·0
C 30	6	5·5	9	4·9	5 weaned	23·5	—	—	—	—

* Lactation not observed following 1st gestation.
† In all 2nd and 3rd gestations, litters were reduced to 6 young on day following delivery.
‡ All vitamins and cystine doubled on 13th day of pregnancy.

Results obtained by exchanging the young of the low fat diet mothers with those of stock diet females suggested that milk production on the low fat diet was probably inadequate.

Four male and 4 female second generation animals weaned by females C5, C8 and C30, which had grown up on the low fat diet (Table IV), were kept on this diet for a period of 16 weeks, at which time the average weight for males was 228 g. and for females 165 g. The appearance of these animals was normal.

Following their last gestation, the female rats used for reproduction were continued on the low fat diet for 10–20 weeks. Despite the nutritional strain of pregnancy, and in some cases lactation, they showed no signs of abnormality.

Motile sperms were found in the epididymides of male rats after they had been 44 weeks on the low fat diet.

Feeding experiment on the low fat vitamin E-deficient diet

As the possibility existed that the vitamin E concentrate used in the complete low fat diet might, in addition to vitamin E, contain another factor essential for the rat, 10 male and 8 female rats were reared on the low fat diet without vitamin E. The growth of these animals, though somewhat inferior to that of the controls, gave no indication that any factor other than vitamin E was lacking. The average growth curves are given in Fig. 1. The average time when weight became stationary for the 8 females was 16 weeks, and for 8 of the males 23 weeks. Two of the males had not ceased growing at 37 weeks. As was the case with the animals on the complete diet, there was an inexplicable tendency in both males and females to resume growth after maintaining a constant weight for 6–10 weeks. This occurred in about half of the animals and the gains varied between 10 and 20 g. in 9–12 weeks.

After the deficiency of vitamin E in the diet had been checked by resorption gestations on several females, 4 female rats whose weights were stationary were given 20–40 mg. of the vitamin E concentrate weekly. An average gain of 18 g. in 12 weeks resulted, as contrasted with an average gain of 1 g. for their 4 controls. This gain corresponds rather closely to the difference between the growth curves of female rats with and without vitamin E.

The oestrous cycle was followed on 6 rats from the 8th to the 23rd week of the experiment. The average length of the cycle for 5 of the rats was 4·7 days. For the 6th animal the average was 5·8 days. The prolongation of the cycles in this case occurred during postoestrus.

At the 40th to the 42nd week of the experiment some of the vitamin E-deficient animals showed the first symptoms of the paralysis mentioned by Evans [1932] and later described by Blumberg [1935], Ringsted [1935] and Burr *et al.* [1937]. There was an unmistakable spreading of the hind legs accompanied by a lowering of the posterior abdominal region. This abnormality was observed in 5 of 8 males and 1 of 4 females. Thirteen male and 5 female rats on the low fat diet with added vitamin E concentrate showed no signs of the paralysis at 42–44 weeks.

Discussion

Inspection of the "fat-free" diets used by others in nutritional investigations, and consideration of the analytical findings reported in this paper, show that in addition to the known fat-soluble dietary essentials such diets have included light petroleum-soluble substances of unknown significance. The diet used in this experiment contained at the most, exclusive of the fat-soluble essentials, 0·0156 % of lipid. Of the fat-soluble essentials, vitamin E alone was added in an impure form, and here the contamination did not exceed 7 mg. of lipid matter per rat per week. The disadvantages resulting from the use of a concentrate instead of the pure vitamin were partially mitigated by running a parallel experiment on the low fat diet without vitamin E, thus reducing the non-vitamin lipid to 0·0056 %. The symptoms of avitaminosis-E would hardly be expected to obscure an additional nutritional deficiency.

The growth of animals maintained on the complete low fat diet for as long as 11 months was good and compared favourably with growth on the stock diet. Modifying the low fat diet, even to the extent of replacing it by the stock diet, did not significantly affect the weights of the animals. The differences between the average growth curves of animals on the complete and vitamin E-deficient diets suggested an uncomplicated vitamin E deficiency. Furthermore, the

response of vitamin E-deficient females to the vitamin E concentrate corresponded to the difference between the growth curves and was of the order of magnitude of the growth response obtained by Evans *et al.* [1938] with pure vitamin E. These results, obtained on the complete and vitamin E-deficient low fat diets, furnish no evidence for the existence of an additional fat-soluble growth factor.

Female rats on the experimental diet were fertile. Since it is not likely that the vitamin E concentrate carried a second factor necessary for reproduction, additional evidence is furnished against the existence of a hitherto unrecognized fat-soluble essential. The subsequent normal behaviour of the females used for reproduction and the fair growth of second generation animals point in the same direction. However, negative results in an experiment of this kind can never be conclusive. While the probability of the existence of an additional fat-soluble growth (and to a lesser degree reproduction) factor has been considerably reduced, it must be borne in mind that experiments in which different basal diets are employed may yield opposite results.

It seems probable that the inferiority of young born to females on the low fat diet was primarily due to inadequate milk production. If this was the case, a subminimal supply of the vitamin B complex can be excluded as the cause, since good lactation was obtained in animals used for assay of the yeast extract. One or more of the fat-soluble vitamins may have been involved, though doubling them did not rectify the condition. Similar results on low fat diets have been reported by Evans *et al.* [1934], and Olcott & Mattill [1937]. The former workers found the addition of 25 % lard or butter fat beneficial. While this suggests that a fat-soluble factor, or fat as such, is necessary for normal lactation, in none of these cases has the complicity of cystine or methionine, shown by Daggs & Tomboulian [1935] and Daggs & Lidfeldt [1938] to be potent lactagogues, been excluded.

The failure of Olcott & Mattill [1937] to observe the paralysis reported by Evans [1932], Blumberg [1935], Ringsted [1935], and Burr *et al.* [1937] was probably due to the comparatively short duration of their experiments. On the low fat vitamin E-deficient diet used in this experiment, the first symptoms of the paralysis were observed in some of the animals at 40–42 weeks. Heretofore the evidence that the paralysis is a result of vitamin E-deficiency has rested on the preventive action of wheat germ oil. The absence of symptoms from control animals receiving a highly potent vitamin E concentrate is an advance in the identification of the active substance.

Summary

1. Evidence has been presented indicating the presence of fat in several components of "fat-free" diets.

2. The preparation of a diet lower in lipids than any previously reported has been described. The total lipid content of this diet is approximately 0·27 %. The maximum non-vitamin lipid content of the diet is 0·0156 %, and of the diet without vitamin E concentrate, 0·0056 %. The other fat-soluble essentials were added in pure form.

3. This low fat diet was found satisfactory for good growth and reproduction.

4. The results of feeding experiments on the complete and vitamin E-deficient diets furnish no evidence for the existence of a hitherto unknown fat-soluble factor necessary for growth or reproduction in the rat.

5. The early symptoms of paralysis observed in adult rats on a vitamin E-deficient diet were prevented by a highly potent concentrate of vitamin E.

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CXVI. THE BIOCHEMISTRY OF SILICIC ACID
VIII. THE DETERMINATION OF SILICA

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THE method employed for the determination of silica in tissues and body fluids must depend on the amount and nature of the silica present. For tissues such as the lungs of workers in dusty atmospheres, where a great deal of mineral matter may be present, the ordinary gravimetric procedures are applicable. In the case of normal tissues of low silica content a micro-gravimetric method is suitable [Morgan & King, 1932], but a colorimetric method may be preferred because of the absence of the technical difficulties inherent in the micro-gravimetric procedure. The quantities of silica normally encountered in urine and blood are so small as usually to demand a colorimetric method.

The silica of lungs and lymph glands consists largely of particles of mineral dust which have gained access to the tissues through the air passages. Much of it, being derived from refractory silicate rocks, is insoluble in chemical reagents (e.g. quartz, mica). This material must be decomposed before it can be estimated. For this reason it is essential to fuse the tissue, or its ash, with sodium carbonate. The endogenous "silica of constitution" of other tissues is more easily brought into solution. But it is found most convenient to fuse these also with sodium carbonate, when there can be no question that all of the silica in the tissue is being determined. In blood and urine all the silica appears to be in a soluble state. This makes possible a direct determination through a chemical reaction leading to the production of a colour without the necessity of preliminary decomposition with sodium carbonate.

Several alterations and improvements have been made in the methods of silica analysis employed in this investigation since the publication of the first paper of the series [King & Stantial, 1933] on the "micro-determination of silica". These are outlined in the present communication, together with directions for the application of the method to a variety of biological materials, both normal and pathological, and to mineral dusts. The description of a gravimetric procedure is likewise given.

Methods

Preparation of tissue for analysis. There is a large degree of variation in the silica content of different parts of some tissues. This is notably the case with lungs, where the inhaled siliceous dust settles in largest amount in the region of the hilum, and becomes concentrated in the root glands. With these tissues it is essential to obtain as representative samples as is possible, so that the analysis may represent the composition of the whole organ; wherever possible the entire organ should be taken [cf. Collins & Dible, 1935]. Tissues like liver and muscle are much more uniform in composition, and need not be sampled so carefully. The analysis is preferably done on dried and powdered tissue rather than on fresh tissue. A block of tissue or the whole organ is trimmed free from adherent material, and is washed first with tap water and then with distilled water. It is

cut in small pieces and placed on metal trays in an oven at 105° to dry. After 24 hr. in the oven it will usually have reached constant weight. The hard lumps of dried tissue are pounded to a powder in an iron mortar and again left in the oven overnight. If a power-driven mortar and pestle are available, the reduction of the sample to a fine powder is an easy matter. If it has to be done by hand, the process is facilitated by screening off the finest material several times during the grinding. The powdered dry tissue is finally thoroughly mixed on a sheet of paper, a portion is taken for analysis and the remainder stored in a closely stoppered bottle or in envelopes in a desiccator.

Gravimetric analysis—semi-micro-method (for tissues having a high silica content). The lungs of workers who have been exposed to the inhalation of silica dust may contain 0·5–5 % or more silica on the dry weight. An amount of the dry powdered tissue is taken which may be expected to yield 5–50 mg. SiO_2. The sample is intimately mixed with 4 times its weight of anhydrous Na_2CO_3 in a platinum crucible of 10–20 ml. capacity. The mixture is carefully "smoked off" and heating continued until a clear liquid melt is obtained. The cooled contents of the crucible are dissolved in water on the steam bath. Conc. HCl is carefully added until the mixture is acid to methyl orange, and 3 or 4 ml. extra added. The mixture is evaporated to dryness on the steam bath, and heated for 1 hr. at 105° in the oven to dehydrate the silica. The residue is moistened with a few drops of water and HCl, and again heated for 1 hr. at 105°.

The dehydrated silica is separated from the other constituents of the residue by bringing the latter into solution with dil. HCl. The residue is moistened with conc. HCl and the crucible three-quarters filled with water. The mixture is warmed on the steam bath and stirred with a glass rod. When solution appears to be complete, the crucible is removed from the bath and allowed to stand for a few minutes for the silica precipitate to settle.

Filtration of the silica is accomplished without removing it from the crucible by means of the filter stick illustrated in Fig. 1. In this way it is possible to complete all the operations involved in the analysis in the original vessel without

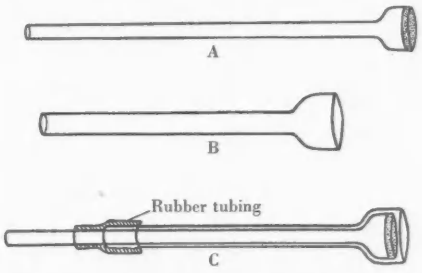

Fig. 1. Special filter stick for silica analysis. A, sintered glass filter stick. B, jacket. C, assembled filtering apparatus. (Obtainable from Messrs Gallenkamp.)

transferring the precipitate. This makes for greater accuracy in the method by avoiding the possibilities of error involved in transference. Filtration by sucking off the filtrate and washing is, moreover, faster than filtration by gravity. As a filtering medium, filter paper pulp shaken up with water, or powdered anthracene is suitable. The latter is easily eliminated by mild heating, whereas paper must be destroyed by ignition. The filtering medium is packed tightly into the cavity formed at the flared end of the filtering apparatus. A piece of fine rubber

suction tubing connects the filter stick to a small suction flask. Suction may be applied by means of a water pump, or, preferably, by mouth with a piece of rubber tubing carrying a pinch-cock attached to the side arm of the flask.

The filter stick is held so that its filtering surface is just below the level of the liquid in the crucible, and the filtrate sucked off under mild suction. The crucible is filled again with warm dil. HCl and the precipitate stirred, allowed to settle, and the supernatant again sucked off. Washing is repeated once with dil. HCl and twice with water. The precipitate is finally sucked dry in the bottom of the crucible.

The plug of filtering medium is dropped out of the filtering apparatus into the crucible by pushing the inner tube of the apparatus (the filter stick) a few mm. through the rubber collar which attaches it to the outer tube or jacket. Particles of silica adhering to the glass are washed into the crucible with alcohol or water from a wash bottle. Any liquid in the crucible is now evaporated on the water bath. The crucible is dried in an oven, or carefully with a flame, and then slowly ignited to red heat, cooled in a desiccator and weighed. HF (5 ml.) is added and a few drops of H_2SO_4. The crucible is gently heated to evaporate off the HF and SiF_4, and then more strongly to drive off the H_2SO_4. It is then ignited, cooled and weighed. The loss in weight on treatment with HF is taken as the weight of silica. Some typical results obtained by this method are given in Table I.

Table I. *Gravimetric analyses of silicotic tissues*

Tissue		Wt. of dry sample g.	mg. SiO_2 found	% SiO_2
Spleen 108	(1)	2·000	10·2	0·51
	(2)	2·000	10·9	0·54
	(3)	2·000	10·6	0·53
Lung 109	(1)	0·250	2·2	0·88
	(2)	1·000	9·2	0·92
	(3)	2·000	18·3	0·91
Lung 110	(1)	2·000	16·0	0·80
	(2)	2·000	16·2	0·81
Lung 111	(1)	1·000	13·4	1·34
	(2)	2·000	28·2	1·41
Lung 116		2·127	142·7	6·71
Lung 121	(1)	1·030	17·2	1·67
	(2)	1·103	17·6	1·60

The colorimetric determination of silica

The colorimetric method adopted for the determination of silica in tissues consists in the production of a yellow silicomolybdic acid complex. This colour may be utilized as such, or it may be reduced by 1:2:4-aminonaphtholsulphonic acid to give a blue colour. Both colours are proportional to the amount of silica present [King, 1926; King & Stantial, 1933] and can be compared colorimetrically with those produced in standard solutions containing a known amount of silica.

The method is applicable to much smaller amounts of silica-containing material and can be executed in a fraction of the time that is necessary for gravimetric determinations. If the procedure is rigidly followed and due precautions are taken to insure the purity of the reagents used and to eliminate interfering substances, the results obtained are nearly as accurate as those given by the gravimetric procedure.

The yellow colour method is much less sensitive than the blue colour method. It may be used with any materials having a high silica content, such as silicotic tissue, lungs and lymph glands. The sensitive blue colour method may be used for any material, but is especially suitable for those of low silica content, normal tissues, blood, urine etc. The yellow silicomolybdic acid colour is preferably read in a photoelectric colorimeter, a photometer, or in Nessler or Hehner tubes. It is difficult to match in a Duboscq colorimeter. The reduced silicomolybdic acid blue colour can be assessed in any type of colorimeter; it is easily read in a Duboscq instrument. The comparison is greatly facilitated and made much more accurate if made in the light of a sodium lamp, such as those supplied with the Hellige and Klett instruments.

Several substances interfere with the reaction giving the silicomolybdic blue colour. Of these phosphate and iron are the most important in biological material. Phosphate gives a blue colour in the presence of molybdic acid and aminonaphtholsulphonic acid at all acidities. As phosphorus is an invariable constituent of animal tissues, its removal is necessary before the silica can be estimated. The presence of iron in the solution leads to the development of a greenish tint, and the colour can neither be matched against a simple silicate standard, nor is it proportional to the amount of silica present. In the original method the phosphate and iron were removed in two stages, the iron by precipitation with excess ammonium dihydrogen phosphate and the phosphate by precipitation with calcium chloride and ammonia.

The simultaneous elimination of phosphate and iron from a silicate-containing solution is accomplished by the basic ferric acetate procedure of Jacobs [1931], which was previously used for the preparation of a protein- and phosphate-free filtrate of blood. By this means the alkaline precipitation with ammonia is avoided and the precipitation of phosphate can therefore be carried out in glass vessels. Moreover, both phosphate and iron are precipitated in one step instead of the two which were previously necessary. The method removes phosphates as ferric phosphate, excess iron as basic ferric acetate and any colloidal or suspended material which may be present, such as protein, fat and debris (and in the case of urine, part of the pigment).

Solutions and reagents

Silica standard. Sodium silicofluoride. The most satisfactory standard used thus far is a solution of sodium silicofluoride. This salt is a light anhydrous non-hygroscopic powder which hydrolyses in solution to give silicic acid. 314 mg. of the solid in 1 l. of water give a concentration equivalent to 1 mg. SiO_2 in 10 ml. ("strong standard"). A "weak standard" (0·1 mg. SiO_2/10 ml.) is made by diluting the "strong standard" 1 in 10. It has been found advisable to make these fresh each week.

10 N H_2SO_4. 278 ml. of conc. acid to 1 l. with water.

N H_2SO_4. 1 in 10 dilution of 10 *N*.

N NaOH. The NaOH, to be silica-free, must be prepared from the metal. 2·3 g. metallic sodium are dissolved in 100 ml. of water in a nickle crucible, and preserved in a wax bottle. B.D.H. "sodium hydroxide pure (from sodium)" is satisfactory. Glass pipettes should be brought into the NaOH solution only for the briefest possible times.

Ferric chloride in hydrochloric acid. 1 % $FeCl_3$, $6H_2O$ in 0·02 *N* HCl.

Sodium acetate in sodium hydroxide. 1·5% $C_2H_3O_2Na$, $3H_2O$ in 0·028 *N* NaOH. The ferric chloride and sodium acetate solutions, when mixed in equal amount, diluted with water, heated and filtered, yield a fluid of approximately *p*H 6.

Acid molybdate. 5 % ammonium molybdate in N H_2SO_4.

Reducing agent. 0·2 % 1:2:4-aminonaphtholsulphonic acid in 2·4 % Na_2SO_3, $7H_2O$ and 12 % $NaHSO_3$.

Ammonium phosphate solution. 2 % $(NH_4)H_2PO_4$.

Calcium chloride. 2 % $CaCl_2$.

Perchloric acid. 60 % $HClO_4$.

Acetic acid. Glacial and 10 %.

Potassium ferrocyanide. 5 % aqueous solution.

Pyramidon. 1 % aqueous solution.

The original article should be consulted for details of the preparation of solutions, and for the precautions which must be observed in the use of the method. Frequent "blank" determinations should be carried out on the several solutions to determine that they are silica-free. Only chemical reagents of analytical grade should be used.

The preservation of fluids for analysis should always be in non-glass vessels. Urine and blood which are kept in glass increase in silica content. The dissolution of silica from the glass can be retarded by the addition of acid to the fluid; this is only possible in the case of urine. The Lusteroid test-tubes made of heavy cellophane by the International Equipment Co. of Boston, U.S.A. (and sold by Messrs Gallenkamp) are satisfactory for the collection and preservation of fluids for silica analyses.

Tissue analysis: 0·1–0·5 g. (depending on its silica content) of dry tissue is weighed into a platinum crucible or dish (preferably of large size, 25–50 ml.). Anhydrous Na_2CO_3 equal to about four times the weight of tissue is added and the mixture carefully heated. After the sample has been "smoked off" destruction of the remaining organic matter takes place smoothly and rapidly in the melting mass. The heating is continued until a clear melt is obtained. Water (about 10 ml.) is added to the cooled crucible, and the fused material dissolved by heating on the steam bath. When solution is complete the crucible is cooled, and its contents washed through a funnel into a 100 ml. volumetric flask. The solution is carefully neutralized with N H_2SO_4, using Congo red[1] as indicator, and shaken vigorously the while to rid the mixture of CO_2. The titration is facilitated by first neutralizing to phenolphthalein with $10N$ H_2SO_4 and then finishing the titration with Congo red and N H_2SO_4. Approximately the same amount of acid is necessary for the second part of the titration as for the first. 20 ml. each of ferric chloride and sodium acetate solutions are now added. The volume is adjusted to 100 ml., the mixture shaken and poured into a 250 ml. conical flask. It is brought rapidly to the boiling point with vigorous shaking. By this means the iron and phosphate are completely removed as ferric phosphate and basic ferric acetate. The hot mixture is poured on a folded 30 or 32 Whatman filter paper. A water-clear filtrate is obtained which is suitable for the colorimetric silica estimation.

In order to determine that the solution is now phosphate-free, 1 ml. of the cooled filtrate should be tested for $PO_4^{\equiv}$ ion by the addition of 2 drops of $10N$ H_2SO_4, 2 drops of molybdate and 1 of reducing agent. The development of any blue colour indicates the presence of phosphate, and the basic ferric acetate precipitation should be repeated by heating 50 ml. of the filtrate with 10 ml. of ferric chloride and of sodium acetate.

Congo red is preferred as indicator because it is completely removed in the subsequent precipitation with basic ferric acetate. Other indicators, e.g. methyl orange, are not so removed, and make the final colorimetry difficult or impossible.

A second 1 ml. portion of the filtrate is tested for iron by the addition of 2 drops of potassium ferrocyanide solution and a drop of dilute acetic acid. Careful neutralization of the solution before the ferric acetate treatment usually ensures that no phosphate or iron will come through in the filtrate.

Yellow colour method

An aliquot portion of the filtrate—10, 25 or 50 ml. according to the amount of silica expected—is placed in a 50 ml. flask or cylinder. For standards 2, 5 and 10 ml. of "strong standard" (0·2, 0·5 and 1·0 mg. SiO_2) are transferred to 50 ml. flasks. The volumes of tests and standards are adjusted to 50 ml., and 4 ml. of acid molybdate are added to each. The yellow colours are compared after 10 min. in Nessler or Hehner tubes, or in a photometer or photoelectric colorimeter.

Blue colour method

An amount of the filtrate which may be expected to contain from 0·02 to 0·12 mg. SiO_2 is transferred to a 25 ml. volumetric flask. 2, 5 and 10 ml. portions of the "weak standard" (equivalent to 0·02, 0·05 and 0·1 mg. SiO_2) are pipetted into similar flasks. Water is added to about 20 ml., and then 2 ml. of acid molybdate and 0·5 ml. of reducing agent. The flasks are shaken, and kept for 10 min. for the colours to develop. 10*N* H_2SO_4 (1 ml.) is then added to each flask to stabilize the blue colours, the volumes are adjusted to 25 ml. and the contents of the flasks thoroughly mixed. Comparison of the test is then made against the appropriate standard.

Notes on tissue analysis. Lung and thoracic and mesenteric lymph nodes of adults are usually much richer in silica than other tissues and small samples should be used for analysis. 100 mg. (or less) of a silicotic lung powder or of peribronchial and mediastinal lymph nodes give sufficient silica for colorimetric determinations (lung tissue and lymph glands from young persons or animals however require the larger amounts).

Brain and nerve tissues have higher phosphate contents than most other tissues and here 25 ml. of the precipitating reagents should be used. (Sufficient of the reagents should be added before boiling to provide an excess of iron which is indicated by the formation of the brown precipitate of basic ferric acetate. If insufficient iron is present only white ferric phosphate is formed and the filtrate will contain at least a trace of phosphate when tested.)

Bone, which is largely calcium phosphate, is difficult to ash, and more sodium carbonate is necessary, the melt never becoming clear. After the fusion mixture has been taken up in water and neutralized, the precipitate of calcium phosphate should be removed by filtration, the precipitate and filter paper being washed thoroughly to remove all silica. The filtrate still contains much phosphate and 25 ml. each of the ferric chloride and sodium acetate precipitating reagents may be required.

Calcified lymph nodes present the same difficulties as bone and should be treated in a similar manner.

A comparison of results obtained by the yellow and blue colour methods and by gravimetric analysis is given in Tables II and III.

Table II. *Comparison of silica analyses by different methods*

Tissue	Blue colorimetric % SiO_2	Gravimetric % SiO_2
Spleen 108, abrasive soap powder worker	0·54	0·53
Lung 109, silica brick grinder	0·85	0·90
Lung 110, coal miner	0·82	0·80
Lung 112, coal miner	1·45	1·45
Lung 116, fuller's earth worker	6·70	6·71
Lung 121, coal miner	1·34	1·53

Table III. *Comparison of results of tissue analyses by yellow and blue colorimetric procedures*

Lymph glands	Age in years	Yellow colour method	Blue colour method
228, housewife	43	0·98	0·98
257, traveller	60	1·33	1·34
259, labourer	41	1·23	1·26
271, housewife	73	1·37	1·37
275, housewife	86	1·78	1·75
286, pensioner	79	0·64	0·63
289, housewife	46	1·07	1·03
290, taxi-driver	61	0·93	0·95
293, laundress	50	1·05	1·00
394, policeman	80	0·64	0·64
582, motor driver	67	0·61	0·62
586, housewife	73	2·38	2·41
597, housewife	87	1·58	1·57
601, dairyworker	44	1·33	1·35
606, housewife	62	3·27	3·33
643, pensioner	74	1·37	1·35
663, hairdresser	62	0·53	0·54

Urine analysis

5 ml. of human urine (or of the urine of carnivorous animals) are treated with 5 ml. of water and 20 ml. each of ferric chloride and sodium acetate solutions. 1 or 2 ml. of the urine of herbivorous animals, which contains a much higher concentration of silica, are sufficient. The mixture is heated rapidly to boiling in a conical flask, and poured hot on the filter paper.

The filtrate should be clear and practically colourless. It is tested for phosphate and iron. If either be found, a fresh specimen of urine should be carefully acidified with acetic acid (Congo red indicator) and the precipitation repeated. 10 or 20 ml. of filtrate are transferred to 25 ml. volumetric flasks, and 2, 5 and 10 ml. portions of "weak standard". The solutions are treated as previously described to develop the blue colours.

Table IV. *Colorimetric analysis of urine—recovery of added silica*

	mg. SiO_2 per 100 ml.	SiO_2 added, mg. per 100 ml.	Total SiO_2 found, mg. per 100 ml.	mg. SiO_2 recovered
Synthetic urine	0	0·5	0·5	—
,, ,,	0	3·0	2·98	—
Dog urine	0·53	1·0	1·47	0·94
Sheep urine	17·2	5·0	22·1	4·9
Human urine	0·85	1·0	1·87	1·02
,, ,,	1·56	1·0	2·63	1·07
,, ,,	2·35	1·0	3·32	0·97
,, ,,	2·35	5·0	7·40	5·05
Human urine after ingestion of silicic acid	6·85	1·0	7·87	1·02
	6·85	10·0	17·0	10·15

Blood analysis—soluble silica

Perchloric acid—basic ferric acetate deproteinization. "Direct" method. The deproteinization of blood with basic ferric acetate, which was previously described [King & Stantial, 1933], has not in practice always yielded a completely protein-free filtrate. For this reason a preliminary partial deproteinization with perchloric acid is now recommended. The phosphate in the filtrate is then removed by the basic ferric acetate method. The resulting filtrate contains all the

silica originally present in the blood, as is shown by comparison of the values obtained by this method with those for ashed samples of blood. Measured amounts of silicic acid, added to the blood, have been quantitatively recovered in the filtrate by the colorimetric procedure.

2 ml. of blood (fresh or oxalated—1 drop of 10 % potassium oxalate for 3 ml. blood) are laked with 10 ml. of distilled water. 1 ml. of calcium chloride solution and 0·3 ml. of perchloric acid (60 %) are added. The mixture is shaken and centrifuged. 10 ml. of the supernatant are transferred to a 15 ml. volumetric flask. A drop of phenolphthalein is added, and *N* NaOH to neutralize the perchloric acid. The pink colour is discharged and the solution made faintly acid by a small drop of glacial acetic acid. 1 ml. each of ferric chloride and sodium acetate are added and the volume adjusted to 15 ml. The mixture (transferred to a 50 ml. conical flask) is brought rapidly to the boil with vigorous shaking over a free flame, and is poured, while still hot, on a folded filter.

10 ml. of cooled filtrate (equivalent to 1 ml. of blood) with 1·2 ml. of molybdate and 0·3 ml. of aminonaphtholsulphonic acid are transferred to a 15 ml. volumetric flask for the colorimetric estimation. A 0·01 mg. SiO_2 standard is used.

"Indirect" method by precipitation with pyramidon. King & Watson [1936] found that silica could be completely precipitated from solution as the pyramidon salt of silicomolybdic acid. Advantage of this fact may be taken to concentrate the blue colour in a smaller volume, thereby rendering it more easily assessable in the colorimeter. 10 ml. of filtrate are treated in a conical 15 ml. centrifuge tube with 1 ml. of acid molybdate and 1 ml. of pyramidon solution. The precipitate of pyramidon silicomolybdate is allowed to flocculate during 10 min. in a beaker of water at about 60°, and is then centrifuged down at 3000–4000 r.p.m. for 10 min. The supernatant is carefully poured off and the tube inverted to drain on filter paper for 5 min. The precipitate is dissolved in 0·5 ml. of reducing agent, and diluted with 3 ml. of water and 0·5 ml. of acid molybdate. Standards consisting of 1 ml. of "weak standard" and of 0·5 ml. with 0·5 ml. of water are precipitated with 2 drops each of sodium acetate, ammonium molybdate and pyramidon.

The final 4 ml. of coloured solution obtained by this procedure are of a much more intense blue than the 15 ml. of coloured solution obtained from the same amount of blood in the "direct" method. Because of the small volume of coloured solution it is necessary to use a colorimeter equipped with micro or semi-micro attachments.

Table V. *Colorimetric analyses of blood, recovery of added silica*

	mg. SiO_2 per 100 ml. of blood	mg. SiO_2 added per 100 ml.	Total mg. found per 100 ml.	mg. SiO_2 recovered
Horse serum	1·18	2·0	3·23	2·05
" "	1·18	3·0	4·11	2·93
Defibrinated horse blood	1·27	3·0	4·16	2·89
" " "	1·31	3·0	4·16	2·85
Mixed, oxalated human blood (1)	1·66	1·0	2·60	0·94
(2)	1·47	1·0	2·50	1·03
(3)	1·39	1·0	2·22	0·83
	1·38	1·0	2·25	0·87
(4)	1·14	2·0	2·91	1·77
	1·14	3·0	4·28	3·14
(5)	1·30	3·0	3·96	2·66
(6)	1·39	3·0	4·42	3·03

Blood serum may be treated as in the perchloric acid-basic ferric acetate method for whole blood.

The bloods used in the recovery experiments (Table V) and for comparison of methods (Table VI) were preserved with the ordinary grade of potassium oxalate in the soft glass containers used for the routine collection of blood in the hospital. It will be noted that the figures are higher than those shown in Table VII for samples of blood preserved with analytical grade potassium oxalate, or with heparin, in non-glass vessels.

Table VI. *Comparison of "direct" and "indirect" methods for blood*

	mg. of SiO_2 found per 100 ml. of blood	
	"Direct" method ($HClO_4$-FeOAc precipitation of proteins)	"Indirect" method (pyramidon-molybdate precipitation of SiO_2 from filtrate)
Oxalated human blood	1·30	1·39
	1·27	1·31
	0·93	1·00
	1·06	1·12
	2·63	2·63
	1·50	1·42
	1·42	1·47
	1·03	1·13
Blood to which sodium silicate has been added	2·10	2·17
	2·69	2·73
	4·16	4·16
	3·76	3·94
	3·48	3·61

Table VII. *Silica of human blood*

	mg. SiO_2 per 100 ml. of blood	
Blood preserved in glass	1·11	1·13
,, ,,	0·89	0·90
,, ,,	0·84	0·84
Fresh blood (post-prandial)	0·47	0·45
,, (1 hr. post-prandial)	0·45	0·46
,, (3 hr. ,, ,,)	0·43	0·42
Heparinized blood, in cellophane tubes	0·62	0·63
,, ,, ,,	0·53	0·55
,, ,, ,,	0·30	0·33
Oxalated blood, in cellophane tubes	0·60	0·62
,, ,, ,,	0·32	0·30
,, ,, ,,	0·42	0·35

Ascitic fluid and C.S.F.

Fluids such as these, of low protein content, are best treated by a direct deproteinization (with simultaneous elimination of phosphate) with basic ferric acetate [King & McGeorge, 1938]. 3 ml. portions of the fluid are treated in 100 ml. conical flasks with 6 ml. of ferric chloride and 6 ml. of sodium acetate solution. The mixtures are brought quickly to the boil, with vigorous shaking, and filtered hot. 10 ml. of filtrate in 15 ml. flasks are treated as for blood filtrates.

Total silica of blood

5 ml. of blood with 1 ml. of ammonium phosphate solution[1] are carefully dried in a platinum crucible. 0·5 g. Na_2CO_3 is used for the ashing and the analysis carried out as in the case of "Tissue Analysis".

Sputum analysis

Because of the presence of mineral particles in the sputa of individuals who have inhaled siliceous dust [cf. Burke, 1935], the analysis of the sputum for silica may occasionally be desirable. The analysis is best carried out on a weighed sample; because of its viscous nature sputum is not conveniently measured with a pipette. The samples are collected in waxed paper containers, and poured into weighed platinum crucibles. The wet weight is recorded, and the dry weight after drying at 105°. Na_2CO_3 is added, the sample is ashed, and the analysis completed as for tissues. Results are probably better expressed as a percentage of the dry weight, since the water content of sputum varies greatly.

Determination of silica in mineral dusts

It is frequently necessary, in investigations related to silicosis, to analyse samples of rock or of rock-dust for their total silica content. The gravimetric analysis will usually be preferred, but in special circumstances, e.g. with small samples of air-borne dust, the colorimetric procedure may be necessary. If the mineral specimen is known to contain no phosphorus and no iron, it should be fused with Na_2CO_3, dissolved in water, brought to an appropriate volume and a suitable amount taken for colorimetric analysis. Most industrial dusts, however, contain iron. Ferric silicate is insoluble in an alkaline medium, viz. the solution of the Na_2CO_3 melt, and although hydrolysed by acid it may not be recovered on neutralization of the Na_2CO_3 solution because of its sticking to the walls of the platinum crucible. In order to avoid loss of silica through precipitation as iron silicate it is necessary to add sufficient phosphate to insure the complete removal of the iron as insoluble ferric phosphate. The excess phosphate is removed in the subsequent treatment with ferric chloride and sodium acetate.

An amount of mineral dust sufficient to contain 1–10 mg. SiO_2 is fused with Na_2CO_3. 1 ml. of phosphate solution is added and water to dissolve the melt. The subsequent procedure is the same as for tissues. Some results are shown in Table VIII.

Table VIII. *Analysis of mineral dusts. Colorimetric method*

	Weight of sample mg.	% SiO_2 found	% SiO_2 found by gravimetric analysis
Quartz	10	97·0	96·6
Kieselguhr	5	87·7	88·3
	50	88·2	—
Flint	10	90·1	89·0
Stone dust	5	52·3	54·9

[1] See the section on "Mineral Dusts", in connexion with the necessity of adding extra phosphate in the case of materials containing an excess of iron over the phosphorus present.

SUMMARY

Methods for the determination of silica in tissues and body fluids are described. Directions are given for a gravimetric procedure using a modified filter stick, and for colorimetric procedures which employ the silicomolybdic acid yellow colour and the reduced silicomolybdate blue colour.

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CXVII. INVESTIGATIONS ON THE POLYPEPTIDE-CONTENT OF THE SERUM

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Existing methods for determination of the polypeptide content of serum are mainly based on two different principles. According to the first the protein is removed and estimations are made with the protein-free filtrates. It is thus essential that the protein precipitant should not precipitate any polypeptides. In such a protein-free filtrate the amino-nitrogen may be determined before and after hydrolysis, the difference is taken as an index for the polypeptide content [Hiller & Van Slyke, 1922; Becher & Herrmann, 1931, 1, 2; 1932, 1, 2; Martens 1930].

In the second type of method two different protein-free filtrates of the serum are made, of which the first contains the polypeptides whereas the second filtrate does not. The difference in some chemical constituent is taken as an index of the polypeptide content, e.g. the nitrogen content [Hahn, 1921; Wolff, 1921; Puech & Cristol, 1929] or the tyrosine content [Goiffon & Spaey, 1934].

Both principles have been strongly criticized. It is the object of this paper to demonstrate that under the correct circumstances reliable results may be obtained with the aid of a method based on the second.

Choice of method

The method used was that of Goiffon & Spaey, the proteins being first removed from the serum with the aid of two different protein precipitants.

The first filtrate is obtained by mixing 1 vol. serum with 9 vol. 3% trichloroacetic acid; after standing for at least 15 min. the solution is filtered. It will be shown that this solution still contains the polypeptides. The second filtrate is obtained by mixing 1 vol. serum with 9 vol. of a solution containing phosphotungstic acid (22 g.) and *N* HCl (30 ml.) in 1 l.; again after at least 15 min. the solution is filtered. This time the filtrate will not contain the polypeptides. Hence the filtrates will have different nitrogen and tyrosine contents, and the difference between the nitrogen content of the trichloroacetic acid filtrate (TN) and that of the phosphotungstic acid filtrate (PWN) will correspond to the nitrogen content of the polypeptides, the so-called "double nitrogen" (DN). 5 ml. of each filtrate are treated with two drops of the phenol reagent of Folin and Denis[1] and 4 ml. of 10% Na_2CO_3; after 30–60 min. one drop of saturated aqueous Na_2SO_3 is added and the volume made up with water to 10 ml. Comparison is made with 5 ml. of a standard solution of tyrosine, containing 0·01 g. tyrosine in 1 l. of 0·1 *N* HCl similarly treated. From the difference in colour the

[1] This reagent was prepared by dissolving 25 g. of sodium molybdate and 100 g. of sodium tungstate in 700 ml. of water; 50 ml. of 85% phosphoric acid and 100 ml. of 38% hydrochloric acid were added; after boiling for 8 hr. under reflux the solution was cooled and the volume adjusted to 1 l.

tyrosine index may be calculated which indicates the number of mg. of tyrosine contained in the polypeptides of 1 l. of serum. In order to prove that the double nitrogen and the tyrosine index really do depend on the presence of polypeptides, a peptidase (erepsin) was added to a number of sera; by this means the values of the double nitrogen and of the tyrosine index were reduced almost to zero. Care had to be taken that the erepsin preparation was free from proteases.

In the first experiments a commercial erepsin was used which proved useless since the preparation itself gave a strong colour with the reagents used for the determination of the tyrosine index; moreover, a few hours after its addition the serum proteins began to be broken down. The commercial preparation itself contained nitrogen compounds which were not precipitated by trichloroacetic acid, but were precipitated by phosphotungstic acid.

Erepsin was therefore prepared according to Waldschmidt-Leitz & Schäffner [1926]. 100 g. of the mucosa of the small intestine of the pig were ground up with 500 ml. of 87% glycerol. For each experiment 10–15 ml. of this suspension were mixed with 3 vol. water and centrifuged. The supernatant fluid, containing the erepsin, was precipitated with about 2 ml. 0·1 *N* acetic acid. The tube was inverted in order to remove the fluid as completely as possible. The precipitate was mixed with the serum, of which TN, PWN, DN and the tyrosine-index (TI) had just been estimated. The precipitate soon settled down. Estimations of DN and TI were carried out immediately and after several hours at 37° [Godfried, 1938]. Table I shows some of the results.

Table I

Exp. no. ...	5			6			7			8		
		Serum + erepsin			Serum + erepsin			Serum + erepsin			Serum + erepsin	
	Serum	At once	After 18 hr.	Serum	At once	After 10 hr.	Serum	At once	After 10 hr.	Serum	At once	After 10 hr.
TN	51·2	50·7	50·1	56·3	56·3	56·3	45·1	45·6	46·8	59·1	59·2	59·9
PWN	30·0	30·0	50·1	50·7	50·2	55·8	38·1	37·6	46·2	57·2	57·3	59·9
DN	21·2	20·7	0	5·6	6·1	0·5	7·0	8·0	0·6	1·9	1·9	0
TI	38	36	2	18	18	2	18	18	0	—	—	—

Exp. no. ...	9			10				11		
		Serum + erepsin			Serum + erepsin				Serum + erepsin	
	Serum	At once	After 3 hr.	Serum	At once	After 6 hr.	After 30 hr.	Serum	At once	After 5 hr.
TN	18·5	18·6	18·5	143·1	142·0	143·1	142·5	31·1	36·7	37·5
PWN	13·2	13·4	18·5	116·8	116·8	124·3	140·6	24·9	30·5	36·1
DN	5·3	5·2	0	26·3	25·2	18·8	1·9	6·2	6·2	1·4
TI	22	22	0	46	46	32	4	26	26	0

TN = trichloroacetic acid filtrate-N; PWN = phosphotungstic acid filtrate-N; DN = polypeptide-N all in mg./100 ml.; TI = tyrosine index.

From these experiments the conclusion may be drawn that the double nitrogen and the tyrosine index correspond to polypeptides which are broken down by erepsin.

In a further experiment glycyl-*l*-tyrosine and *d*-leucylglycine were added to serum; the nitrogen of these compounds could be recovered quantitatively in the trichloroacetic acid filtrate, but was absent from the phosphotungstic acid filtrate.

The results obtained by the tyrosine method did not always run parallel with those obtained by the nitrogen estimations, presumably owing to the variability of the tyrosine and nitrogen contents of different polypeptides.

Results

According to the tyrosine method the normal value of the polypeptide content of the serum is less than 25 (as mg. of tyrosine per litre of serum); according to the nitrogen method, described above, it is less than 7 mg./100 ml.

Conditions in which the serum content of polypeptides is increased may be divided into three groups. Some of the results are shown in Table II. For more detailed figures the reader is referred to the author's thesis [Godfried, 1938].

The first group contains those cases in which there is increased parenteral destruction of proteins. In gastric and duodenal ulcer hyperpolypeptidaemia has been reported [Marino & Saladino, 1937]; in the writer's experience such hyperpolypeptidaemia was only found shortly after a haemorrhage. Suppuration may be accompanied by hyperpolypeptidaemia [Hülse & Strauss, 1924]. This has been confirmed in the present work and attention is drawn to the increased content of polypeptides in ulcerative colitis. When the condition of a patient suffering from ulcerative colitis improves, the hyperpolypeptidaemia becomes less marked (Table II, no. V[1]). In leukaemia, especially when the patient has been treated with X-rays, hyperpolypeptidaemia may be marked. In malignant new growth, chiefly when extensive metastases exist, very high indices for the polypeptide content may be found. The highest values are reached when metastases have formed in the liver. Among diseases of metabolism the presence of hyperpolypeptidaemia in severe diabetes [Puech, 1926, 1, 2] has been confirmed. In several cases of sprue and of gout an increase in serum polypeptides was also found.

The second group comprises diseases of the liver. It is well known, that under normal conditions polypeptides are broken down to urea or synthesized into proteins in the liver [Fiessinger, 1934]. In cirrhosis of the liver hyperpolypeptidaemia may or may not be found [Labbé & Nepveux, 1931; Becher & Herrmann, 1931, 1, 2; 1932, 1, 2; Valdiguié, 1934; Bentz & Larizza, 1937]. Reference has already been made to the association of malignancy involving metastases in the liver with a high polypeptide content of the serum; the estimation of the degree of the polypeptidaemia in jaundiced patients may indeed be of great value, especially to assess the indication for cholecystogastrostomy in cases of a carcinoma of the head of the pancreas. If such a carcinoma is still small, it may nevertheless cause jaundice; in such cases cholecystogastrostomy may enable the bile to flow away from the liver along the intrahepatic bile passages. If, however, large metastases have formed in the liver, these will obstruct the intrahepatic bile passages and cholecystogastrostomy will not cause the disappearance of the jaundice. If hyperpolypeptidaemia is found in cases of carcinoma of the pancreas, the presence of large metastases is very probable and cholecystogastrostomy will remain without any effect. Cases IX[32] and IX[33] demonstrate this clearly; in both patients, suffering from a carcinoma of the head of the pancreas and of the bile passages respectively, marked jaundice and hyperpolypeptidaemia were present; in both cholecystogastrostomy was performed, but in both it remained without any result. However, even if metastases in the liver exist in a jaundiced patient suffering from a carcinoma of the head of the pancreas, the polypeptide content of the serum may still be low (compare case IX[30]).

The third group is formed by diseases of the kidneys [Pribram & Klein, 1923; Becher & Herrmann, 1931, 1, 2; 1932, 1, 2; Puech & Cristol, 1929; Martens, 1928; Larizza, 1937]. High blood pressure alone is not accompanied by a hyperpolypeptidaemia; acute and chronic nephritis, however, are; apparently the diseased kidney does not excrete sufficient polypeptides. No parallelism exists between the polypeptide content and the non-protein-nitrogen. The polypeptide content of the serum in a patient suffering from chronic nephritis does not give any prognosis of the duration of life, nor does any relation exist between the polypeptide content and the usual kidney function tests (phenosulphophthalein-test, urea clearance etc.). The raised values in cases of cardiac decompensation may be considered to be caused by disturbance of the functions of the liver and of the kidneys.

Table II. *Polypeptide content of serum in various diseases*

No.	Sex	Age	Diagnosis	TI	TN	PWN	DN
III[2]	♂	39	Duodenal ulcer	42	—	—	—*
				22	—	—	—†
III[8]	♂	35	Haemorrhage from a gastric ulcer	52	—	—	—
IV[2]	♂	69	Pneumonia	50	—	—	—
IV[6]	♀	30	Bronchiectasis	54	—	—	—
IV[8]	♂	24	Abscess of the lung	62	32·5	9·8	22·7
V[1]	♀	28	Ulcerative colitis	70	—	—	—
				30	35·6	28·8	6·8‡
V[2]	♀	26	Do.	42	—	—	—
V[3]	♀	11	Do.	104	—	—	—
V[9]	♀	23	Do.	46	22·4	11·5	10·9
V[12]	♀	35	Do.	44	71·4	61·3	10·1
V[13]	♂	51	Do.	44	33·0	16·2	16·8
VI[2]	♂	49	Lymphatic leukaemia	34	63·6	54·0	9·6
VI[5]	♀	50	Do.	86	—	—	—§
VII[4]	♂	64	Tumour of the lung	128	—	—	—
VII[8]	♀	59	Metastases of a carcinoma of the chest	36	29·7	18·7	11·0
VIII[1]	♂	47	Diabetes	80	—	—	—
VIII[2]	♀	40	Do.	88	—	—	—
VIII[12]	♂	53	Gout	80	—	—	—
VIII[6]	♀	53	Sprue	68	—	—	—
VIII[7]	♂	65	Do.	40	30·5	20·4	10·1
VIII[9]	♂	51	Do.	—	51·8	30·0	21·1
IX[2]	♀	57	Cirrhosis of the liver	100	—	—	—
IX[9]	♂	67	Cirrhosis of the liver; gall stones	44	23·0	11·8	11·2
IX[11]	♂	26	Do.	4	24·9	23·2	1·7
IX[12]	♀	53	Do.	10	27·4	25·8	1·6
IX[24]	♀	40	Gall stones; abscess of the liver	56	—	—	—
IX[26]	♀	52	Metastases of a carcinoma of the chest	120	—	—	—
IX[30]	♂	48	Carcinoma of the head of the pancreas; metastases in the liver	30	—	—	—‖
IX[31]	♂	52	Do.	124	—	—	—
IX[32]	♀	59	Do.	80	—	—	—‖
IX[33]	♂	60	Carcinoma of the bile passages	138	—	—	—‖
X[3]	♀	46	Hypertension	16	26·6	22·4	4·2
X[5]	♀	54	Do.	26	31·1	24·9	6·2
XI[8]	♂	40	Acute nephritis	56	—	—	—
XI[10]	♀	17	Do.	40	31·1	21·8	9·3
XI[24]	♀	40	Chronic nephritis	50	160·4	144·7	15·7
XI[26]	♀	36	Do.	26	48·7	41·4	7·3
XI[33]	♂	60	Do.	38	21·6	9·5	12·1
XI[34]	♀	21	Do.	40	43·7	30·2	13·5
XI[35]	♀	25	Do.	46	143·1	116·8	26·3
XIII[12]	♂	46	Cardiac decompensation	78	73·9	53·7	20·2
XIII[3]	♀	30	Do.	88	—	—	—

* Immediately after a haemorrhage. † 18 days afterwards.
‡ A year after the first estimation. § Treated with X-rays. ‖ See text.

SUMMARY

It has been shown that the differences in nitrogen content or in tyrosine content of two filtrates of the same serum, obtained after the addition of 3% trichloroacetic acid and phosphotungstic acid respectively, correspond to the polypeptides of the serum. Hence these differences form a reliable index of the polypeptide content of the serum. The circumstances where the serum contains excessive amounts of polypeptides are discussed.

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CXVIII. PORPHYRINURIC ACTION OF DRUGS RELATED TO SULPHANILAMIDE. COMPARISON WITH REPORTED TOXICITY, THERAPEUTIC EFFICIENCY AND CAUSATION OF METHAEMOGLOBINAEMIA. DEFINITION OF THE STRUCTURE RESPONSIBLE FOR PORPHYRINURIC ACTION

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It has been shown [Rimington & Hemmings, 1938] that a marked increase in porphyrin excretion follows the daily oral administration to white rats of sulphanilamide at dosage levels of 0·4–1·5 g./kg. body weight. The porphyrins were isolated from urine and faeces and identified as coproporphyrin III together with small quantities of coproporphyrin I. Porphyrinuria due to these same two pigments was demonstrated in a series of human clinical cases undergoing a course of sulphanilamide treatment, and similar observations have more recently been reported by Long & Bliss [1938], and also by Silver & Elliott [1939].

Discussion of the mechanism of this porphyrinuria led to the conclusion that the drug, in the doses employed, exerts a toxic action most probably affecting both the liver and the bone marrow. The possibility was indicated that some patients might prove to be more susceptible than others and it was suggested that determination of the amount of porphyrinuria might serve as a means of assessing the toxic action of sulphanilamide upon this phase of haematopoietic activity and of revealing individual idiosyncrasy. That sulphanilamide is capable of causing profound disturbances in the pigment metabolism of the body is evident from the frequency with which cyanosis accompanied by demonstrable methaemoglobinaemia has been reported. Thus, in a review of the types of toxic manifestation occurring in 408 patients exhibiting sulphanilamide intoxication, Long *et al.* [1939] noted cyanosis in 90–100%, whilst most observers agree that at least half of all their patients treated with the drug show some degree of cyanosis. There has been much discussion as to whether methaemoglobin is alone responsible, or whether products derived from sulphanilamide may also contribute to the discoloration. Some observers have gone so far as to assert that methaemoglobinaemia is of comparatively rare occurrence; on the other hand, careful investigations by Lockwood *et al.* [1938], Hartmann *et al.* [1938] and others have shown that a close correspondence can be traced between the degree of cyanosis and the quantity of spectrophotometrically determined methaemoglobin. It cannot be too greatly emphasized that in particular those toxic effects revealed by changes in the pigment economy and metabolism of the organism are deserving of much more thorough investigation than has hitherto been accorded them [compare Rimington, 1939].

In the present communication, experiments are described by means of which answers to the following questions have been sought:

(1) In treatment with the sulphonamide series of drugs, is porphyrinuric activity an invariable accompaniment of therapeutic effectiveness or does this symptom parallel toxicity to the animal as a whole (minimal acute fatal dose)?

(2) Which chemical grouping or portion of the molecule is responsible for porphyrinuric activity?

(3) Is there any relation between methaemoglobin formation (compare sulphanilamide cyanosis) and increased excretion of porphyrin?

A series of substances related to sulphanilamide or possessing certain of the structural elements of this drug have been fed over periods of 1, 2 or 3 weeks to rats maintained, as in the previous experiments, upon a sufficient, synthetic diet. The doses were varied to allow of comparison as to porphyrinuric effect, and in all cases when a toxic action was manifest, post-mortem histological examinations have been made. The results demonstrate that porphyrinuric action is closely associated with ability to produce methaemoglobinaemia and runs roughly parallel with toxicity as a whole.

Experimental

Technique of animal experiments

Certain improvements in technique have been made since the publication of our first paper, and our present method will therefore be recorded in detail.

White rats weighing 150–200 g. were kept in groups of three in metabolism cages arranged for the separate collection of urine and faeces. These cages were made of metal, the funnel-shaped lower portion being carefully treated with molten paraffin wax at intervals of about a week, to prevent any possibility of the urine coming into direct contact with the metal surface. Toluene was added to the glass urine-collecting flasks.

The artificial diet was made up as follows, and was fed *ad lib.* Water was supplied *ad lib.* from Drummond bulbs:

Synthetic diet. Casein, 20%; rice starch, 73%; salt mixture, 2%; yeast powder, 5%; cod-liver oil, 20 μl. per rat per week.

Salt mixture. NaCl, 41·6 g.; $MgSO_4$, $7H_2O$, 130·2 g.; NaH_2PO_4, 83·2 g.; K_2HPO_4, 228·8 g.; $CaHPO_4$, 129·6 g.; ferric citrate, 47·2 g.; calcium lactate, 146·2 g.

The food was not placed in the metabolism cages, but the animals were removed and allowed to feed and drink in separate cages for 1 hr. in the morning and 1 hr. in the evening (a single period of 2 hr. on Sundays). A small quantity of urine was unavoidably lost during this time, but contamination of the collecting receptacle with debris was obviated. All animals were weighed at weekly intervals. The drugs were administered orally, unless otherwise stated, usually in the form of a suspension in 10% gum acacia solution. A requisite volume of this mixture was introduced daily into the oesophagus by means of a hypodermic syringe fitted with a blunted metal needle. Liquid drugs were introduced into the oesophagus in the same manner. Groups of normal rats, kept under similar conditions, served as controls.

Determination of urinary porphyrin

The urine sample, representing a 3-day collection from three rats (about 70 ml.), was filtered through glass wool into a separating funnel and the collecting flask rinsed with 20–30 ml. of water. To the combined filtrates were added 20 ml. of glacial acetic acid followed by 200 ml. of ether. The mixture was well shaken and the lower aqueous layer was run off as far as the small

gelatinous emulsion which is almost invariably found at the interface. Half an hour was allowed for this emulsion to contract into a more solid mass, from which the ether was decanted. The emulsion residue was now shaken again with 3–4 ml. of acetic acid and 100 ml. of ether, this solvent, after decantation, being used for a final extraction of the main bulk of the urine. The combined ether extracts were washed four times with 200 ml. of water, the first two washings containing 5 ml. of saturated potassium acetate solution adjusted, if necessary, to *p*H 7·0. After removal of the bulk of the dissolved acetic acid from the ether phase in this way, the porphyrins were extracted by shaking with successive lots of 10 ml. of 5% (by weight) HCl, allowing the acid phase to settle down completely each time before tapping off. Completion of extraction can be controlled by spectroscopic or fluoroscopic examination. Usually three or four extractions suffice to remove all the porphyrin. Such an acid extract will only contain the porphyrin in very great dilution and may also contain other colouring matters which interfere with quantitative determination, especially if a fluorescence method be used. It has been our practice, therefore, to purify and concentrate the porphyrin as follows.

To the 5% HCl extract in a smaller separating funnel are added 5 ml. of ether and sufficient saturated potassium acetate to render the reaction neutral to Congo red. (8 ml. are sufficient for 30 ml. of acid extract.) After vigorous shaking, the ether is transferred to a 20 ml. capacity funnel having a very short outlet tube, and the aqueous solution is extracted with two more successive 5 ml. lots of ether, which are combined with the first. This ether solution is washed once with 3 ml. of water, taking care to shake down drops adhering to the glass. The coproporphyrin is then extracted by shaking with 0·5 ml. lots of 0·5% by weight HCl until the acid absorption spectrum is no longer visible. Usually five shakings suffice to remove even as much as 100 μg. The volume is then carefully adjusted to 3 ml. (or more, if much porphyrin is present) and the quantity of coproporphyrin present determined by measuring the light absorption in the region of the β band of the acid porphyrin spectrum (centre about 549mμ.) on a Hilger-Nutting spectrophotometer previously calibrated against standard coproporphyrin solutions. Since the relation between porphyrin concentration and the photometer scale reading is linear, the exact concentration of porphyrin in the test solution is readily obtained. Concentrations as low as 3 μg./ml. in the final solution are easily determined by this method.

It will be noticed that we have employed 0·5% HCl for the final extraction of the coproporphyrin. The advantage of this procedure is that any protoporphyrin present through accidental contamination of the urine specimen with faeces is thereby left behind in the ether, since protoporphyrin has a much higher acid number than has coproporphyrin.

The rats selected for any particular experiment were usually allowed to remain for 6–12 days upon the basal synthetic diet, whilst a "base line" of porphyrin excretion was being obtained. Daily dosing was thereafter commenced and continued for 9–24 days, according to the promptness of response. In many instances, the effect did not become very marked until about the ninth day, after which porphyrin excretion rose rapidly. It is possible that saturation of the organism with the drug, or the precursor of the porphyrin, occurs during the initial period, since the phenomenon of delayed response was particularly striking in the case of substances like sulphanilamide and proseptasine which are poorly absorbed and less evident or absent when materials like aniline or hydroxylamine were dosed. It is also possible that a hepatotoxic effect might develop during the course of some days [compare Espersen, 1937].

At the end of an experiment, the animals were killed by coal gas and specimens of liver, spleen and kidney (sometimes also bone-marrow) taken in corrosive-acetic fixative for histological examination.

All preparations were examined for stainable iron (prussian blue reaction) in addition to haematoxylin-eosin differentiation.

Isolation of the porphyrins

The urine extracts from experiments with any one drug were pooled and the ether-soluble porphyrins isolated in the following manner.

The 0·5% HCl solution was filtered into a small separating funnel and covered with a layer of ether. A few drops of glacial acetic acid were then added and sufficient saturated aqueous potassium acetate to render the mixture neutral to Congo red. Upon shaking, the porphyrins passed up into the ether layer which was separated, washed well by shaking with water and allowed to evaporate to dryness. The residue was washed once with light petroleum and then esterified by dissolving in methyl alcoholic HCl (saturated at 0°). After 24 hr., chloroform was added and much water in a separating funnel. The chloroform layer, containing the porphyrin esters, was washed well with dilute Na_2CO_3 and then water and evaporated to dryness. After rinsing with light petroleum, the dry residue was treated with cold, anhydrous ether until no more pigment passed into solution. This ether solution, containing the coproporphyrin III ester, was evaporated to a very small volume and set aside in a closed tube in the ice-box for crystallization. Any ether-insoluble pigment was dissolved in a little chloroform and to the concentrated solution about 5 vol. of hot methyl alcohol were added and the mixture set in the ice-chest. Any coproporphyrin I ester separating could be centrifuged down, but in many cases the quantity present was barely more than a trace, too small for identification by melting-point. The crystals of coproporphyrin III ester, which formed in the concentrated ether solution, were separated from mother liquor (removing the latter with a micro-pipette), washed sparingly with cold, anhydrous ether, then with light petroleum and dried over P_2O_5 *in vacuo*. Melting-points were recorded on the Kofler electrically-heated micro-melting-point apparatus.

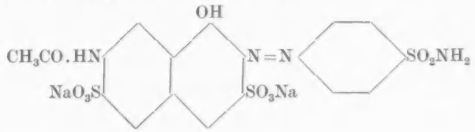
Results

Economy of space prevents the full details being given for each substance tested. The data for experiments with sulphanilamide and aniline are given in full in the protocol in the Appendix; all the findings are presented in summary form in Tables I and II.

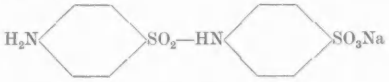
The following drugs were investigated:

(1) *p*-Aminobenzenesulphonamide ("sulphanilamide"):

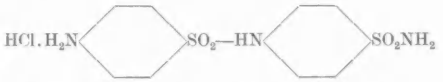
(2) Disodium 4′-sulphonamidophenyl-2-azo-7-acetamino-1-hydroxynaphthalene-3:6-disulphonate ("prontosil soluble"):



(3) Sodium *p*-aminobenzenesulphonyl-*p'*-aminobenzenesulphonate ("sodium sulphanilyl-sulphanilate"):

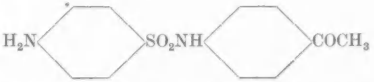


(4) *p*-Aminobenzenesulphonyl-*p'*-aminobenzenesulphonamide hydrochloride ("disulphanilamide hydrochloride"):



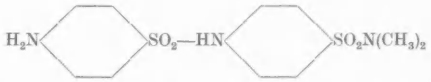
(5) *p*-Benzylaminobenzenesulphonamide ("proseptasine"):

(6) 4-Sulphanilylamidoacetophenone:

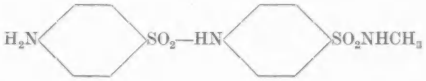


This substance was kindly placed at our disposal by the manufacturers, Messrs E. Merck, in consultation with the Therapeutic Trials Committee of the Medical Research Council. We are grateful to Messrs Merck and to the above-named Committee for permission to publish our findings.

(7) *p*-Aminobenzenesulphonyl-*p'*-aminobenzenedimethylsulphonamide ("uliron"):



(8) *p*-Aminobenzenesulphonyl-*p'*-aminobenzenemethylsulphonamide ("diseptal B"):



(9) 4:4'-Diaminodiphenylsulphone:

(10) 4:4'-Diacetaminodiphenylsulphone:

(11) Sulphanilic acid.

(12) *p*-Aminobenzoic acid.

(13) *p*-Phenylenediamine.

(14) Aniline, purified by redistillation.

(15) Monomethylaniline (prepared by hydrolysis of the crystalline acetyl compound, "exalgin", and purified by redistillation).

(16) Dimethylaniline (purified by repeated recrystallization and finally redistilled).

(17) 2:4:6-Trichloroaniline.

(18) Phenetidine (purified by redistillation).

(19) Benzidine.

(20) *o*-Toluidine.

(21) 5-Chloro-*o*-toluidine.

(22) Nitrobenzene (purified by redistillation).

(23) Methylacetanilide ("exalgin").

(24) Hydroxylamine hydrochloride.

(25) Phenylhydroxylamine (freshly prepared by Imperial Chemical Industries and stored in N_2 in the dark).

(26) Sodium nitrite.

(27) *iso*Amylamine.

Discussion

A discussion of our results will be much facilitated by reference to the structural formulae on pp. 963–4 and to Tables I and II in which the main findings are summarized.

It will be seen that porphyrinuric activity is by no means limited to sulphanilamide, but is shared by most of the drugs belonging to the sulphonamide series. In addition, a number of the simple aromatic amines tested, particularly aniline and its derivatives, were found to possess this property to a marked degree.

No doubt, were it possible to give sufficiently high doses, it would be found that a great number of substances would cause porphyrinuria, but on the basis of the dosage rates employed in this investigation it would seem permissible to draw certain general conclusions, relating porphyrinuric action and therapeutic efficiency to chemical constitution, as follows.

(1) *There is no quantitative relation between therapeutic efficiency and porphyrinuric action.* Within the series of sulphonamide drugs, it is not always easy to compare therapeutic action with toxicity on account of the fact that many of the substances are sparingly soluble and poorly absorbed so that only a small proportion of the ingested dose actually finds its way into the blood stream. Nevertheless, substances like sulphanilamide and disulphanilamide, for example, are considered to exert comparable therapeutic effects and yet the latter drug does not cause any increase in porphyrin excretion in the dosage attainable. Similarly, many of the simpler substances related to the sulphonamides have been shown to increase very markedly the urinary porphyrin excretion and yet they are devoid of therapeutic action in experimental infections.

(2) *In general, porphyrinuric action runs roughly parallel with general toxicity.* An apparent exception to this generalization is the case of 4:4′-diacetaminodiphenylsulphone, which is tolerated in single doses >40 g./kg. but which produces quite a pronounced porphyrinuria when administered daily at the level of 0·18 g./kg. These amines and their acetyl derivatives are known to come into a state of dynamic equilibrium *in vivo* and hence one might expect the toxicity of the diacetyl derivative of the sulphone to be much greater (approaching that of the parent amine) when given regularly than when administered on a single occasion [compare Nitti *et al.* 1938]. The post-mortem histological changes suggest a similar action. Moreover, it must again be pointed out that the

Table I

No.	Drug	M.Pro.D.* g./kg.	M.L.D.† g./kg.	Porphyrinuric action: Dose g./kg.	Porphyrin excretion	(× normal)	M.P. and re-M.P. of Me ester of porphyrin isolated	MetHb formation	P.M. histological findings	Remarks
1	Sulphanilamide	0·25–1	4	0·4	+	2·5	142–4°,	+	Liver, small amount of stainable iron and some brownish pigment. Kidney, some brownish pigment in convoluted tubules. Spleen, intensely engorged, much stainable iron, also brownish pigment	There was a marked increase in water intake and in urine vol. accompanied by loss in body wt.
				0·93	+	4	166°			
				1·23	+	5				
				1·39	+ +	7				
				1·42	+ +	6·5				
				1·5	+ + +	8				
2	Prontosil soluble	1	4 (i.m.‡)	1·25 (i.m.)	+ +	6	146°,	+	Spleens of groups on larger dose were engorged, contained brown amorphous pigment and stainable iron. Remaining organs and second group normal	
				2·10	+ + +	13	168°			
3	Na sulphanilyl-sulphanilate	s.c.§ ineffective; *per os* 2	20	0·05	–			–		Slight loss in body wt. Urine vol. unchanged. Slight local reaction at site of s.c. injection
				0·25	–					
				1·0 (s.c.)	–					
4	Disulphanilamide hydrochloride	0·25–1	20	0·53	–			–		Animals gained in wt. High M.L.D. and absence of porphyrinuric action, probably due to poor absorption with resulting low concentration in blood [Feinstone *et al.* 1938]
				0·80	–					

5	Proseptasine	0·5	20	1·0	+	2			Slight engorgement of spleen with some deposits of stainable iron	
6	4-Sulphanilyl-amidoaceto-phenone	0·5	>10	0·55 1·15	+ + + +	7·6 7·1			Much brown pigment and stainable iron in spleen	Loss of body wt. Urine vol. unaltered
7	Uliron			1·0	±			−		Marked loss in wt. and moderate increase in urine vol. Coats became rough and staring
8	Diseptal B			1·0	+	3				Wt. and urine vol. unchanged. Uliron has caused fatal funicular degeneration with an inflammatory reaction of the spinal cord [Schubert, 1938]. Both nos. 7 and 8 cause polyneuritis [Hüllstrung & Krause, 1938, 1, 2] but cyanosis only infrequently
9	4:4′-Diaminodiphenylsulphone	0·0025 –0·01	0·25–0·5‖	0·028 0·045 0·080 0·15	+ + + + Hburia + + (deaths)	3·5 3·5	140–3°, 170–2°, also trace of ether-insoluble ester	+	Small deposits of stainable iron in the spleen, but in general much less marked than in the case of sulphanilamide	Marked loss in body wt. Hyperexcitability and loss of hair. Cyanosis very marked
10	4:4′-Diacetaminodiphenylsulphone	0·25–0·1	>40	0·18 0·59	+ +	4 5	142°	+	Slight deposit of stainable iron in spleen	Much less toxic than no. 9

* M.Pro.D. = Minimal protective dose, mouse or rat.
† M.L.D. = Minimal lethal dose, mouse or rat.
‡ i.m. = Intramuscularly.
§ s.c. = Subcutaneously.
‖ Repeated doses.

Table II

No.	Substance	Porphyrinuric action: Dose g./kg.	Porphyrinuric action: Porphyrin excretion	Porphyrinuric action: (× normal)	Porphyrinuric action: M.P. and re-M.P. of Me ester of porphyrin isolated	MetHb formation	P.M. histological findings	Remarks
11	Sulphanilic acid	1·4 1·8	– –			–		Smaller dose administered as Na salt, dissolved in drinking water. Lack of effect and of therapeutic activity may be due to high solubility or to dipole nature of the molecule
12	*p*-Aminobenzoic acid	1·25	–			–		Substance also a dipole
13	*p*-Phenylene-diamine	0·18 0·27	± ±			–		Causes dermatitis. The urine became very dark in colour
14	Aniline	0·35 0·59	+ + + +	3 12	139°	+	Spleens engorged; slight increase in stainable iron. One liver contained a little stainable iron	Well-known cause of methaemoglobinaemia and of bladder tumours. Porphyrinuric action not previously discovered. Urine vol. increased by about 60%, very dark in colour but Hb absent. Nicotinic acid (0·1 g. per rat per day) had no effect on the porphyrinuria
15	Monomethyl-aniline	0·025 0·125	+ + +	3 5·5	133–7°	+	Slight engorgement of spleen, but no abnormal quantity of stainable iron	
16	Dimethylaniline	0·054 0·26	± +	1·6 1·8	158°	+	Fairly abundant deposits of brown pigment in spleen and moderate increase in stainable iron	Dimethylaniline can be demethylated *in vivo* by the dog, cat and rabbit [Horn, 1936, 1, 2], the latter, however, only removing one Me group
17	2:4:6-Trichloro-aniline	0·01 0·054 0·625	– ± +			–	No increase in stainable iron in either liver, spleen or kidney	Substance dosed dissolved in olive oil. Both *o*- and *p*-positions are occupied thereby, preventing oxidation *in vivo* to an *o*- or *p*-iminoquinone
18	Phenetidine	0·044 0·22	+ +	2 3·5	137°	+	Engorgement of spleen but no increase in stainable iron	

19	Benzidine	0·25	+ +	7·5	153°	+	Engorgement of spleen with fairly abundant deposits of stainable iron. Some also present in liver	Well known to be a cause of cyanosis and intoxication among industrial workers, who inhale the light powdered substance
20	*o*-Toluidine	0·11	+	3·5		+	Slight deposition of stainable iron in spleen	
21	5-Chloro-*o*-toluidine	0·15	+	3·5		+	Some vacuolation of liver cells. Spleen engorged and contained much stainable iron	Substance dosed dissolved in olive oil. Causes both cyanosis and dermatitis in man. All animals lost wt.
22	Nitrobenzene	0·125	+	2	146°, 170°	+		Well known to cause cyanosis and "anilism" among industrial workers. The nitro compounds can be reduced *in vivo* to the corresponding hydroxylamines or amines
23	Methylacetanilide	0·11 0·13	– –			–		Animals exhibited nervous excitability and twitching. The drug differs from acetanilide in that having the amino group completely substituted, it does not cause methaemoglobinaemia
24	Hydroxylamine-hydrochloride	0·075 0·15	+ + +	4·5	145°, 170-1°	+	Liver and kidneys contained brownish black particles in which the iron stain was positive. Spleens engorged, but otherwise normal	The substance is an active haemolytic agent [de Langen & Grotepass, 1938]
25	Phenylhydroxylamine	0·014	+	3·3		+		Animals markedly cyanosed. This substance or a derivative is postulated as an intermediate in the oxidation of all the amines here investigated (see Discussion)
26	Na nitrite	0·18	–			+	Liver showed engorgement with vascular dilatation. Otherwise organs normal	Administered 10 mg. *per os* daily and a 0·4% solution in the drinking water. Porphyrin excretion decreased to nearly nil. Urine colour normal. Possibly some degree of anaemia
27	*iso*Amylamine	0·11	–			–		

figures quoted by various workers for the minimal fatal dose of these sparingly soluble compounds are apt to be misleading. The quantity of drug reported as being tolerated in a single dose may sound impressive, but in all probability the greater part of the material is not absorbed.

(3) *In general, all those substances found to exert a porphyrinuric action are also capable of producing methaemoglobinaemia* in vivo and

(4) one can therefore define the chemical grouping necessary for increasing porphyrin excretion in the same terms as allotted to the structure giving rise to methaemoglobin formation—viz. *the presence of an aromatic amino group, unsubstituted or potentially free and preferably in such a system that it is capable of undergoing oxidation with the formation of a hydroxylamine derivative or a reversibly oxidizing system such as a* p- *or* o-*iminoquinone.*

The role of the primary amino group in sulphanilamide in determining the toxicity of this substance has already been suspected [Jennings & Southwell-Sander, 1937], largely as a result of the work of Kracke & Parker [1933–4].

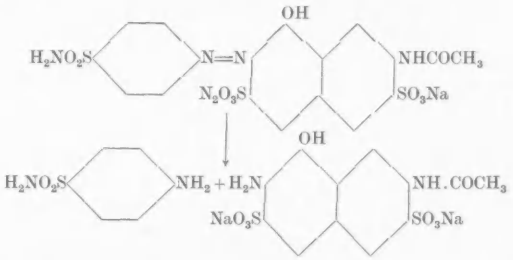
More recently, a striking occurrence reported by Rogers [1938] would appear to support the idea that the amino group is the structural feature, possessed by the sulphonamide drugs in common with certain other substances, which is largely responsible for their specific toxic effects. A woman of 58 was receiving sulphanilamide treatment for erysipelas. Some years previously the local anaesthetic "procain" (*p*-aminobenzoyldiethylaminoethanol hydrochloride) had been administered with evident indications of sensitization. After ingestion of as little as 1·6 g. of sulphanilamide, reactions occurred at all the old sites of injection in a roughly quantitative manner. Patch tests were performed and proved consistently positive to procain but not to sulphanilamide. The author, however, concluded that the feature which these chemical substances exhibited in common was the possession of an aromatic primary amino group. It appears to the writers unfortunate that patch tests were not tried also with phenylhydroxylamine and *p*-aminophenol.

It will be seen, when conclusion no. 4 is examined more carefully with reference to the summarized results in Table I, that not only is there a qualitative correspondence between methaemoglobin production and porphyrinuric action, but that the relationship is also roughly quantitative. Thus aniline, *o*-toluidine and benzidine are well known by experience to cause severe methaemoglobinaemia and they also cause a prompt rise in porphyrin excretion at a relatively low level of dosage. Dimethylaniline, although fully substituted, yet causes a mild degree of methaemoglobinaemia [compare Horn, 1936, 2], and even most carefully purified material was also found to be effective in causing a relatively weak porphyrinuria. *p*-Phenylenediamine, in spite of its tendency to cause dermatitis etc., induces neither methaemoglobin formation nor porphyrinuria. The drug "exalgin" (methylacetanilide) is similarly ineffective, and 2:4:6-trichloroaniline, in which both *p*- and *o*-positions are substituted, is practically ineffective in both respects. The finding with nitrobenzene must also be mentioned. The nitro-compounds are known easily to suffer reduction in the animal body and give rise to methaemoglobinaemia just as do the corresponding amines; in fact the nitro-compounds are perhaps somewhat more dangerous. In the animal experiment it was found by us that so low a dose of nitrobenzene as 0·125 g./kg. brought about a distinct and prompt porphyrinuria. It will be noticed that prontosil is relatively more effective in causing increased porphyrin excretion than is sulphanilamide, when the doses are compared upon the basis of potential equivalence in sulphanilamide content. Prontosil is known to be

transformed, in part at least, into sulphanilamide in the animal body [Fuller, 1937; Jauerneck & Gueffroy, 1937] and could theoretically yield 29·27% of its weight of sulphanilamide.

	Dosage g./kg.	Sulphanilamide equiv. g./kg.	Increase in porphyrin times normal
Prontosil soluble	1·25	0·366	6
	2·1	0·62	13
Sulphanilamide	0·4	0·4	2·5
	0·93	0·93	4

The transformation in the living organism would involve a reduction of the azo linkage giving, in addition, another amino-compound thus:

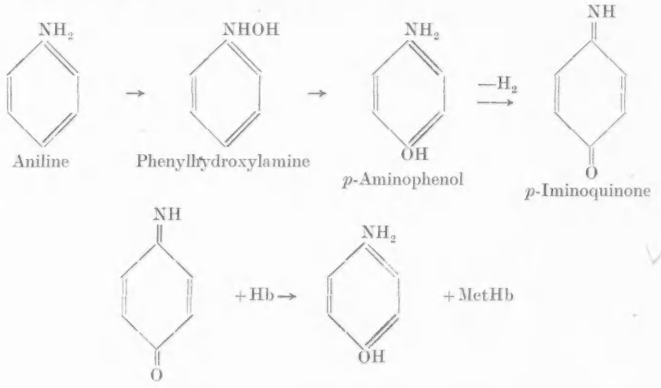


Since one of the conclusions reached as a result of the present investigation is that aromatic amino compounds are potentially capable of producing increased porphyrin excretion, the greater magnitude of the effect produced by prontosil soluble may be due to the fact that both the sulphanilamide and the naphthylamine derivative produced during its biological fission contribute towards porphyrin excretion and the net result is therefore additive. Attention, however, must also be drawn to the fact that the prontosil was given intramuscularly and the sulphanilamide by mouth.

The discovery of this relationship between methaemoglobin-forming properties and porphyrinuric action would seem to be of considerable significance. Not only does it define the chemical structure in the drug responsible for the effect and thereby throw open the possibility of synthesizing a compound free from this adverse characteristic, but possibly still capable of exerting the required chemotherapeutic effects (attempts in this direction have already been made by Michaux *et al.* [1938] who prepared the Na methylenesulphonate derivative of sulphanilamide, and by McLeod [1938]), but it also serves to associate these two manifestations of the toxic action of the sulphonamide drugs upon the haemopoietic system.

Primary aromatic amines, e.g. aniline, can undergo oxidation in the animal body with the formation of phenolic derivatives such as *p*-aminophenol, a reaction in which the hydroxylamine derivative is probably an intermediate. Thus Young & Wilson [1926] were able to detect *p*-aminophenol in the blood plasma and urine after administration of acetanilide to rabbits, whilst Ellinger [1920] recovered acetylphenylhydroxylamine from the blood of acetanilide-poisoned cats, and Lipschitz [1920] has demonstrated the reduction of *m*-dinitrobenzene to *m*-nitrophenylhydroxylamine by minced animal tissues *in vitro*. Heubner, however, does not consider the hydroxylamine to be the actual methaemoglobin-former, since it is irreversibly oxidized *in vivo* with ease to

azoxybenzene [Heubner *et al.* 1923], and his own studies have shown, in agreement with the work of others, that aniline introduced into the blood stream may bring about the formation of many times its equivalent of methaemoglobin; in fact it appears as if an equilibrium is established when approximately one-third of the total blood pigment has been oxidized. Aminophenol, on the other hand, is known to pass very readily and reversibly into *p*-iminoquinone. This could oxidize one equivalent of haemoglobin into methaemoglobin, becoming reduced in the process, be oxidized again by the tissues and enter into reaction with a further molecule of haemoglobin. It would thus act as a catalyst, converting more and more of the blood pigment into methaemoglobin until an equilibrium was eventually established, or it itself became destroyed. The scheme is illustrated by the following diagram:



Should a similar mechanism be entertained as responsible also for methaemoglobin formation in the case of sulphanilamide and its homologues (as first suggested by Rimington [1939]), then evidence must be adduced that the primary aromatic amino group of these compounds is susceptible to such an *in vivo* oxidation as that postulated. Ottenberg & Fox [1938] have shown that on irradiating dilute aqueous solutions of sulphanilamide with ultra-violet light, a rapid change takes place, the solution becoming first purple-coloured and then yellow-brown. Simultaneously there is a decrease of 30–60% in diazotizable amine, determined by Marshall's method. Ottenberg and Fox did not determine the type of change affecting the amino group (see also Main *et al.* [1938]), but repetition of their experiments has convinced us that oxidation plays a prominent part since (1) no colour change takes place when all air is replaced by nitrogen; (2) aeration during the process of irradiation greatly accelerates and intensifies the reaction; (3) the purple-coloured product is reversibly decolorized by a reducing agent such as sodium hydrosulphite (the yellow-brown is not and would appear to represent a further stage of greater complexity, possibly analogous to the transformation of aniline into aniline black); and lastly, (4) methylene blue may replace oxygen in the system as a hydrogen acceptor. Irradiation of sulphanilamide solution under nitrogen and in presence of dilute methylene blue leads to decoloration of the latter.

From these observations we feel justified in regarding the irradiation phenomenon as one of oxidation in which the amino group is involved.

Levaditi *et al.* [1938], following a similar line of thought, have tested experimentally a variety of substances of the quinonoid type of structure for chemotherapeutic activity. Certain of these, such as quinol, diacetylquinol, *p*-aminophenol etc., had a definite though slight effect in gonococcal and meningococcal infections. Mayer [1938], on the other hand, claims to have demonstrated an antistreptococcal activity in *p*-hydroxylaminobenzenesulphonamide equal to that of sulphanilamide itself. This substance (the simple hydroxylamine corresponding to sulphanilamide) was active *in vitro* as well as *in vivo* and Mayer considers that it is in reality the therapeutic agent formed when sulphanilamide is administered to a patient. His conclusion is supported by the fact that the *p*-nitroderivative, which would be reducible *in vivo* to the hydroxylamine, was also active.

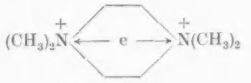
There would appear to be a considerable amount of evidence, therefore, to support the supposition that the amino group of sulphanilamide may undergo oxidation *in vivo*.

Indisputable evidence as to the removal of the sulphonamide grouping *in vivo* is not as yet forthcoming, although on theoretical grounds such a reaction is not improbable. Were these transformations to take place, all the necessary requirements would be fulfilled for the easy production from these drugs of a catalytic oxidizing system capable of forming considerable quantities of methaemoglobin.

Such far-reaching alterations in the molecular structure may not, however. be essential for the manifestation of this activity if the theories put forward by Schwartzenbach & Michaelis [1938] be considered.

These authors have pointed out that certain molecular structures, as exemplified by the indamine and indophenol redox dyestuffs, are capable of electronic rearrangements during a stepwise oxidation, whereby one electron is lost and a partially oxidized or "semiquinone" type of molecule is produced. A resonance system may be set up within the molecule itself, whereby sufficient stability is conferred to allow of the existence of the semiquinone in aqueous solution. Even fully substituted groups, such as the dimethylamino group may participate in this reaction, as illustrated by the simple case of Wurster's blue (see below). Schwartzenbach & Michaelis point out that, so far, all the semiquinones studied contain two equal atoms (other than carbon) symmetrically located in the molecule. They are able to formulate electronically the change of a dyestuff like phenolindophenol from the fully reduced form through the intermediate semiquinone stages to the fully oxidized form without recourse to the more usual benzenoid and quinonoid schemes of representation.

When sulphanilamide is similarly represented, as in the accompanying figure, and its electronic configuration examined, it will be seen that it also could be visualized as suffering stepwise oxidation by the loss of an electron. There appears to be no reason why resonance should not occur throughout the structure from nitrogen to nitrogen, which would materially increase the stability of such a semiquinone type of molecule. On the other hand, were the tissue systems with which it was in contact so adjusted that this intermediate could act as a link in an oxidation-reduction chain, then the requirements for its activity as a catalyst would be met. Depicting haemoglobin as the acceptor, catalytic oxidation to methaemoglobin could occur just as in the case of *p*-aminophenol, previously discussed:



Wurster's blue

Sulphanilamide

The conclusion that oxidation of a primary amino group is responsible for the porphyrinuric action of the sulphonamide drugs, etc., is strongly supported by the finding of Brownlee (personal communication of unpublished results) that a similar increase in urinary porphyrin excretion is caused by another class of pharmaceuticals, namely the antipyretic drugs, phenacetin, phenazone etc., which contain a free or substituted aromatic amino group.

Important though our conclusions concerning the phenomena of methaemoglobin formation and porphyrinuric action appear to us to be for a general understanding of the toxicity of the sulphonamide drugs and their action upon the haemopoietic system, yet we feel that our work would not be complete without some discussion of the possible origin of the extra porphyrin excreted.

Obviously the question which first presents itself is whether excreted coproporphyrin is actually derived from the methaemoglobin of the blood stream. In all instances, the porphyrins isolated after the administration of the drugs we have employed, belonged to the isomeric series III, so that such an origin would be chemically possible. It has, however, become customary to accept the view of haemoglobin degradation put forward by Lemberg [1935] in which the haematin moiety of haemoglobin is pictured as suffering oxidative ring scission whilst the iron and globin are still attached to the molecule and so passing to biliverdin without at any time going through a porphyrin stage. Porphyrins are thus unlikely to arise from the normal breakdown of haemoglobin and there is much physiological evidence to support the correctness of this viewpoint. Recently, however, we have been studying the fate of methaemalbumin formed by injecting haematin into the blood stream of rabbits, man or monkeys and we have evidence that this pigment ultimately appears, in part at least, as extra proto- and copro-porphyrin in the faeces. That haematin injection does not lead to increased bile pigment formation, as does the injection of haemoglobin, has been known for some time [cf. Duesberg, 1933–4]. An increased excretion of coproporphyrin III has also been observed by several workers to occur in certain types of hepatic disease, and in many of these, as reported by Schumm [1927], haematinaemia has been demonstrated.

It would appear, therefore, that not only does there exist the haemoglobin-verdohaemochromogen-biliverdin mechanism of blood pigment degradation, but that, in addition, an alternative route exists by which haematin, the oxidized haem component detached from globin, is metabolized in pathological conditions, at least, but perhaps also normally [compare Thomas, 1938]. It is conceivable that methaemoglobin, if formed in quantity, might follow the same route. Heubner considers that the rate of reconversion of methaemoglobin into haemoglobin *in vivo* is not so great as the earlier experiments of Sakurai [1925] would lead one to suppose.

From histological examination of the organs, and particularly the spleens, of the rats made porphyrinuric by the dosing of sulphanilamide, aniline etc., it is highly probable that an increased red cell destruction has been caused by these drugs. The spleens exhibit a considerable degree of haemosiderosis with dilatation and engorgement [compare Hageman, 1937; Schwartz *et al.* 1938, etc.). Machella & Higgins [1939] have recently demonstrated the production of severe anaemia by the dosing of sulphanilamide to rats.

Tentatively, therefore, we put forward the view that these drugs, capable of suffering oxidation at the amino group *in vivo*, by causing methaemoglobinaemia and increased disintegration of erythrocytes, bring about an increased excretion of type III porphyrins derived from the broken down blood pigment. What role, if any, impairment of normal liver function may play in this sequence of events

we are at present not able to state with certainty. A severe hepatotoxic action of sulphanilamide has been reported in cases by Garvin [1938] and by Cline [1938].

Summary

The finding, already published, that sulphanilamide, when dosed to rats, causes an increase in the urinary and faecal porphyrin excretion, has been confirmed and amplified by extending the investigation to include other drugs of the sulphonamide series and also simpler related chemical substances. From the results which are now reported in detail, with chemical and pathological findings, the following general conclusions may be drawn.

(1) Within the series of sulphonamide drugs tested, there is no quantitative relation between therapeutic efficiency and porphyrinuric action.

(2) Porphyrinuric action runs roughly parallel with general toxicity.

(3) In general, all those substances found to exert a porphyrinuric action are also capable of producing methaemoglobinaemia *in vivo*.

(4) One can therefore define the chemical grouping necessary for increasing porphyrin excretion in the same terms as allotted to the structure giving rise to methaemoglobin formation—viz. *the presence of an aromatic amino group, unsubstituted or potentially free and preferably situated in such a system that it is capable of undergoing oxidation with the formation of a hydroxylamine derivative or a reversibly oxidizing system such as a* p- *or* o-*iminoquinone.*

The last generalization has been discussed in the light of Heubner's work upon the mechanism of methaemoglobin formation following administration of aniline. Both aniline and nitrobenzene (which can suffer reduction *in vivo*) have been shown to be very effective porphyrinuric agents and attention has been drawn to the possible importance of this finding in connexion with the control of the health of industrial workers.

The evidence has been considered which supports the hypothesis that sulphanilamide, etc., may to some extent undergo oxidation at the amino group *in vivo*.

The source of the extra coproporphyrin III excreted as a result of the administration of these substances, has also been considered, and the hypothesis is put forward that it is derived from an increased breakdown of blood pigment. It is suggested that methaemoglobin, when once formed, is degraded, in part at least, by a mechanism which leads ultimately not to bile pigment but to porphyrin. Haematin may possibly represent one of the intermediary stages in this transformation. Further work is in progress designed to elucidate the exact nature of the changes involved.

We wish to thank Dr Buttle, of the Wellcome Physiological Research Laboratories, for generous gifts of some of the drugs and for his kindness in supplying information concerning the effective and fatal doses of some of the compounds investigated. We are also indebted to Dr M. W. Goldblatt and Imperial Chemical Industries for kindly supplying some of the substances used in this investigation, and to Bayer Products, Ltd., for the Prontosil, Uliron and Diseptal B.

APPENDIX

Protocol of experiment with sulphanilamide

Three male rats, av. wt. approximately 200 g., in each group. Groups 36 and 37 and control gained in wt. during the exp., whilst group 38 lost in wt.

	Group 38 (0·93 g./kg.)		Group 36 (1·23 g./kg.)		Group 37 (1·42 g./kg.)		Controls	
	Porph. μg.	Vol. ml.	Porph. μg.	Vol. ml.	Porph. μg.	Vol. ml.	Porph. μg.	Vol. ml.
Pre-period								
i–iii	9·8	40	Trace	50	15·3	52	20·7	23
iv–vi	11·2	62	6	62	5·0	69	30·2	34
vii–ix	10·8	61	13·5	63	10·8	88	29·9	37
x–xii	14·1	66	11·4	53	12·8	66	32·9	31
Days on drug								
1–3	19·1	166	27·2	186	29·9	175	32·7	25
4–6	15·9	97	30·5	182	29·9	131	23·3	27
7–9	46·8	81	35·6	120	40·8	105	30·2	24
10–12	38·6	97	44·0	169	29·1	198	38·7	29
13–15	53·6	82	63·4	169	68·0	124	31·1	29
16–18	45·0	74	64·9	169	62·6	121	—	—

Protocol of experiment with aniline

The average body wt. decreased slightly during the exp.

Group 13. Aniline 0·59 g./kg. per day.

	Urinary porphyrin in μg.			Urine vol. in ml.
	Group 13 3 young ♂		Controls 3 ♀	Group 13
Pre-period (mean)	9		10	73
Days on drug 1–3	110		6	106
4–6	110		7·5	120
7–9	110		7·5	103
10–12	110	Dosed 0·1 g. nicotinic	7·5	137
13–15	130	acid per rat per day	7·5	105

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CXIX. INTERMEDIARY CARBOHYDRATE METABOLISM IN AMPHIBIA

I. CARBOHYDRATE BREAKDOWN BEFORE METAMORPHOSIS

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Carbohydrate metabolism seems to be of dual importance in embryonic life. In the earlier stages of development it is believed that the active substance which brings about neural induction (the evocator) forms a symplex with glycogen and protein and when this is broken down in the dorsal lip of the blastopore, the evocator is liberated [Waddington *et al.* 1935; Waddington *et al.* 1936; Needham, 1936]. In older stages the breakdown of carbohydrate probably provides much of the energy necessary for growth and differentiation.

An extensive study of carbohydrate metabolism in the chick embryo [Needham & Nowiński, 1937; Needham *et al.* 1937; Needham & Lehmann, 1937, 1, 2; Lehmann & Needham, 1937; 1938] showed that the way of carbohydrate breakdown in chick embryo probably differs from that in muscle. The formation of lactic acid in the embryo seems not to pass through a phosphorylating cycle.

The main characteristics of embryonic carbohydrate metabolism found by these authors may be summed up as follows: glycogen is not broken down to lactic acid, whereas glucose and mannose are readily used. The intermediate products of phosphorylation, such as hexose-monophosphate or -diphosphate are not used at all and the same applies to other carbohydrates such as fructose, galactose, sorbose, various pentoses, glucosamine etc. Embryo glucolysis somewhat resembles glucolysis in brain and in tumour tissue. It may be inhibited by *dl*-glyceraldehyde [Needham & Nowiński, 1937] as Mendel [1929] found for tumour tissue. Fluoride also inhibits glucolysis in embryo, but there is no accumulation of hexosediphosphate or phosphoglyceric and glycerophosphoric acids [Needham *et al.* 1937].

These observations on chick embryo seemed sufficiently interesting to warrant the question whether they are typical for embryonic tissue in general. In the present work the carbohydrate breakdown in tadpole tissues (*Rana temporaria*) before metamorphosis has been studied. A later paper will deal with the carbohydrate metabolism during metamorphosis. Studies on mammalian embryo are also planned.

Methods

The methods described previously [Needham & Nowiński, 1937; Needham *et al.* 1937] were used in the present work. Substrate preference and glyceraldehyde inhibition were studied manometrically but in smaller Warburg cups (6·0–7·0 ml.). The temp. of the water bath was 20°. The first reading was taken 20–30 min. after gassing with N_2/CO_2 mixture.

As the younger tadpoles after hatching still contain much yolk and the tissues are saturated with their natural substrate, no significant results could be expected from experiments on this stage. Work was begun, therefore, with stages in which no trace of yolk in the tadpole could be seen under the binocular microscope.

The absolute age of the animals was hard to determine as the eggs were sometimes found already fertilized in the laboratory tanks, but the youngest stages investigated were about 5 days after hatching. The size of the tadpole, as a criterion of age, is not very dependable, but it led to the interesting result that the chemical differentiation (of enzyme systems) of the tissues seems to be independent of growth in length.

If brei is used, the tadpoles being ground in a small mortar until morphological structure is destroyed, the $Q_L^{N_2}$ obtained is so low (for autoglycolysis +1·65, for glucose as substrate +2·85) that the enzyme system is obviously inactivated by the process. Experiments on intact tadpoles were also not very successful as the $Q_L^{N_2}$ for autoglycolysis and glucose as substrate were almost the same (autoglycolysis +12·7 and glucose +13·95). Probably the cutis is not permeable to these substances.

The embryos were therefore pulped with a scalpel, but not into too small pieces. The $Q_L^{N_2}$ thus obtained were more satisfactory (e.g. in Exp. 5 for 10–12 mm. tadpoles: autoglycolysis +10·48; with glucose as substrate +19·2).

The appropriate carbohydrates were added before the experiments started. As the added substances were dissolved in $NaHCO_3$-Ringer for the frog, no dilution occurred of Ringer solution present in the cup.

Autoglycolysis

Table I shows the autoglycolysis of tadpoles during development. In these experiments the total length of the tadpoles was taken as the criterion of age (the figures were mainly obtained from the same lot of tadpoles, measured as development progressed).

Table I

Size of tadpoles in mm.	Average $Q_L^{N_2}$
8–10	+19·35
10–12	+12·7
12–14	+12·1
16–18	+10·72
18–20	+11·9
20–22	+14·6

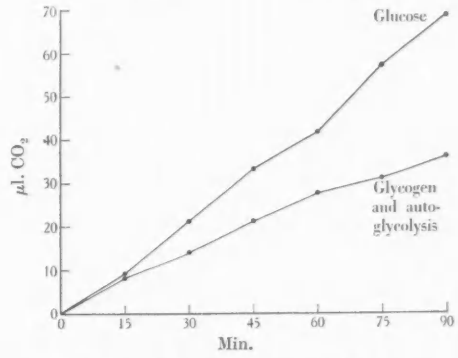

Fig. 1. Preference for glucose. A record of a single experiment.

The curve of autoglycolysis is quite similar to the curve of autoglycolysis in chick embryo [Needham & Nowiński, 1937]: it decreases rapidly with age (Fig. 1). The unusually high $Q_L^{N_2}$ of +14·6 for the 20–22 mm. tadpoles is probably due to "cannibalism": the tadpoles in my experiments were given no food, but in some cases it was impossible to prevent them from eating weak or dying individuals. Fed animals of course grow more quickly but though the size is greater (e.g. 20–22 mm.) the chemical differentiation is just the same as that of 10–12 mm. tadpoles, judged by substrate preference.

Substrate preference

As in the case of chick embryo, glucose but not glycogen is broken down by tadpole tissue. Fig. 1, a record of a single experiment, shows that the curve of glycogen breakdown (in ml. CO_2) runs closely with the curve of autoglycolysis (in all the cups there was approximately the same amount of tissue and the vessel constants differed only slightly), whereas the glucose breakdown gives much higher values, and at the end of the exp., when the glycogen breakdown reaches the value of 36 μl. CO_2, the CO_2 production with glucose as substrate rises to 67·5 μl. The corresponding values for $Q_L^{N_2}$ in this experiment were: autoglycolysis +11·45, glycogen +11·9, glucose +22·5.

These facts were quite reproducible and the main results are collected in Table II. Though there is some scatter among the various experiments, nevertheless the preference for glucose during the course of development of the tadpole is quite obvious.

Table II

Length of tadpoles in mm.	$Q_L^{N_2}$ glycogen	$Q_L^{N_2}$ glucose
8–10	+19·0	+27·0
10–12	+10·48	+19·2
—	+13·5	+22·25
—	+ 8·5	+29·0
—	+15·2	+27·3
12–14	+16·1	—
14–16	+19·2	+30·3
—	+12·0	+21·0
—	+11·26	—
—	+ 7·64	+17·51
16–18	+12·6	—
20–22	+20·25	+25·0

Table III

Exp. no.		$Q_L^{N_2}$
8	Autoglycolysis	+13·5
	Hexosediphosphate	+12·0
	Glucose	+29·0
9	Autoglycolysis	+10·83
	Hexosediphosphate	+10·3
		+10·67
10	Autoglycolysis	+11·5
	Hexosediphosphate	+11·8
		+10·55
		+11·08

Table III shows that adding hexosediphosphate has no effect upon glycolysis, the $Q_L^{N_2}$ for hexosediphosphate corresponding closely to the $Q_L^{N_2}$ of autoglycolysis, whereas glucose (as in Exp. 8) gives a value 70 % higher.

From these results we may draw the conclusion that the mechanism of glucolysis in the tadpole is similar to the glucolysis of the chick embryo.

Glyceraldehyde inhibition

It was first observed by Mendel [1929] that *dl*-glyceraldehyde has the property of inhibiting glucolysis of tumour tissue completely. The same effect may also be produced on the glucolysis of brain [Ashford, 1933], but not on the glycolysis in muscle extract as mentioned by Holmes [1934] and confirmed by other investigators [Needham & Lehmann, 1937, 2; Baker, 1938; Cori *et al.* 1939]. It was therefore suggested that *dl*-glyceraldehyde is a specific inhibitor of glucolysis. As such it was used by Needham and co-workers [Needham & Nowiński, 1937; Needham & Lehmann, 1937, 2] for embryonic tissue and it was found that in chicken embryo glyceraldehyde inhibits the breakdown of glucose. This effect is due to *l*-glyceraldehyde only as was shown by Needham & Lehmann [1937, 2] and confirmed for tumour tissue by Mendel *et al.* [1938]. The specifity of this inhibition was contradicted by Boyland & Boyland [1938] and by Adler *et al.* [1937]. The latter authors claimed to show that glyceraldehyde inhibits not only glucolysis in tumour tissue but also the glycogen breakdown in muscle extract. This discrepancy between the results of these authors and those of Holmes, Needham & Lehmann, Baker and Cori *et al.* was removed by Lehmann & Needham [1938] who showed that freshly prepared glyceraldehyde (in dimeric form) inhibits both ways of carbohydrate breakdown, glucolysis and glycolysis, whereas glyceraldehyde in its monomeric form (produced by standing for some time in solution) acts as a specific inhibitor of glucolysis.

It was of interest to investigate whether tadpole tissue behaves towards this specific inhibitor of glucolysis in the same way as tumour, brain or chick embryo. The results obtained in these experiments showed that it does. Of course stronger concentrations (5×10^{-2} *M*) inhibit glucolysis: in Exp. 25 for instance $Q_L^{N_2}$ for glucose as substrate was +19·8 but for glucose+glyceraldehyde +8·77 only. In another exp., where $Q_L^{N_2}$ for autoglycolysis was +4·55 and for glucose +11·59, the $Q_L^{N_2}$ for glucose+glyceraldehyde was +3·58.

On the other hand Lehmann & Needham [1938] emphasize the importance of a suitable concentration: too strong concentrations may inhibit glycolysis and glucolysis [confirmed by Süllmann, 1939] as in the experiments of Boyland & Boyland and Adler *et al.* Lehmann & Needham obtained maximum inhibition with a glyceraldehyde concentration of $3\text{–}6{\cdot}7 \times 10^{-2}$ *M*. It was therefore necessary to test lower concentrations than stated above.

The experiments were carried out in Warburg manometers. Glucose and glyceraldehyde were added together and another manometer with glucose was used as a control. A further control consisted of tissue without substrate.

The rate of inhibition obtained from these experiments is shown in Fig. 2, being an average from various figures. It is obvious from it that with increasing concentrations the rate of inhibition rises. A glyceraldehyde concentration of

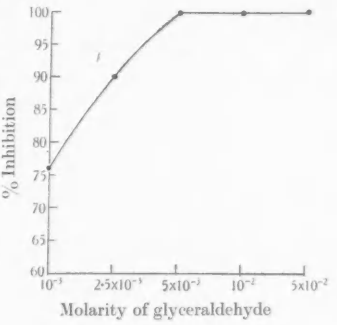

Fig. 2. Inhibition of glucolysis by glyceraldehyde.

10^{-3} *M* inhibits 76 % of glucolysis; $2{\cdot}5 \times 10^{-3}$ *M* inhibits about 90 %; whereas 5×10^{-3} *M* inhibits glucolysis completely. The corresponding figures for $Q_L^{N_2}$ are shown in Table IV.

Table IV

Concentration of glycerald.	$Q_L^{N_2}$ glucose + glycerald.	$Q_L^{N_2}$ glucose	$Q_L^{N_2}$ no substrate
10^{-3} *M*	17·75	34·00	12·65
$2{\cdot}5 \times 10^{-3}$ *M*	10·21	21·15	8·77
5×10^{-3} *M*	6·55	12·30	8·28
10^{-2} *M*	1·70	12·30	8·28
5×10^{-2} *M*	8·79	18·00	8·73

Lehmann & Needham [1938] stated that maximum inhibition has to be produced by a concentration of 3×10^{-3} *M* glyceraldehyde, but not stronger. My results are in accordance with this criterion as the maximum inhibition in my case lay between 5×10^{-3} *M* and $2{\cdot}5 \times 10^{-3}$ *M*. We may, therefore, draw the conclusion that glucolysis in the tadpole resembles that of chick embryo, tumour and brain tissue, in that it may be inhibited by glyceraldehyde.

Triosephosphate accumulation

Needham & Lehmann [1937, 1] showed that in the case of the chick embryo triosephosphate accumulates during the experiment. The following typical experiment shows the method.

Exp. 20. One hundred tadpoles (length 12–15 mm.) were chopped and incubated anaerobically for 1 hr. in Thunberg tubes (some experiments were carried out in Warburg cups). The first tube (X 31) contained tissue without substrate, tube X 52 an appropriate amount of hexosediphosphate and tube X 60 glucose. The amount of tissue was exactly the same in each tube: 2 ml. of brei. After 1 hr. incubation (anaerobically) at 20° the contents of these tubes were ground ice-cold with P-free sand and 3 ml. 3 % trichloroacetic acid, and filtered through asbestos in a Gooch crucible. 1 ml. of the filtrate was removed for estimation of the inorganic P. 1 ml. of filtrate was made alkaline (2*N* NaOH) and after standing for 20 min. at room temp., the P estimation (Fiske-Subbarow) was carried out. The difference of the two estimations gave the amount of triosephosphate.

The results of my experiments are shown in Table V. The differences between the alkali-labile + inorganic P and the inorganic P are very small. They are within the range of colorimetric experimental error. It would therefore appear that hexosediphosphate is not attacked at all by tadpole tissue.

Table V

No. of cup	Substrate	Inorganic P (mg.)	Alkali-labile P + inorganic P (mg.)
X 31	None	0·075	0·055
14	None	0·079	0·080
11*a*	None	0·039	—
X 52	Hexosediphosphate	0·051	0·069
11	Hexosediphosphate	0·054	0·073
13	Hexosediphosphate	0·049	0·058
X 60	Glucose	0·054	0·062
13	Glucose	0·071	0·079
14	Glucose	0·062	0·045

SUMMARY

1. The anaerobic glycolysis of tissues of tadpoles between loss of yolk and metamorphosis was investigated.

2. As in the case of the chick embryo, tadpole autoglycolysis decreases in the course of development.

3. Tadpole tissue shows a preference for glucose; glycogen and hexosediphosphate are not broken down.

4. *dl*-Glyceraldehyde in a concentration 5×10^{-3} *M* inhibits by about 90 % the glycolysis of tadpole tissue.

5. These facts suggest that glucose breakdown in the tadpole does not go through the phosphorylation cycle.

6. After addition of hexosediphosphate no triosephosphate accumulation in the tadpole was observed (in contrast to the chick embryo).

I wish to express my sincerest thanks to Prof. Sir F. G. Hopkins for the hospitality of his laboratory and to Dr J. Needham for his help and advice in carrying out these experiments and for suggesting this problem. I am also much indebted to Dr B. E. Holmes for lending me Warburg manometers with small cups and to Dr D. M. Needham and Dr D. E. Green for hexosediphosphate and *dl*-glyceraldehyde respectively. I am very grateful to Dr H. Lehmann for help in the method of triosephosphate estimation and for some suggestions concerning the glyceraldehyde inhibition.

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CXX. MICRODETERMINATION OF HYPOXANTHINE AND XANTHINE

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In the course of experiments on the formation of hypoxanthine in pigeon liver [see Edson *et al.* 1936] it was found desirable to have a micromethod for the determination of hypoxanthine. In this paper we describe a method based on the use of xanthine oxidase which has proved satisfactory in work on surviving tissues and on enzymes. Hypoxanthine and xanthine are quantitatively oxidized to uric acid, the former requiring 2 atoms of O, the latter 1 atom. If one purine base only is present and if the nature of this is known, its concentration can be calculated from the determination of the O_2 uptake. If both xanthine and hypoxanthine are present their concentrations can be calculated if the uric acid formation is measured in addition to the O_2 uptake.

Preparation of xanthine oxidase

The method requires a highly active preparation of xanthine oxidase. This is prepared in the following way [Dixon & Kodama, 1926]: fresh milk (0·5–1 l.) is clotted with a rennin preparation (Crosse and Blackwell's rennet tablets) and the clot is broken up with a glass rod in order to facilitate the separation of the whey from the curd; the whey is filtered off through muslin, one volume of milk yielding about 0·8 vol. of whey. In each 880 ml. of whey are dissolved 215 g. of solid ammonium sulphate. The solution is allowed to stand for about 30–60 min. until the globulins containing the enzyme have risen to the surface. The underlying almost clear solution is removed by suction and the surface layer amounting to less than one-fifth of the total volume is centrifuged for 10–15 min. The globulins now form a solid floating cake which is transferred to a filter paper and dried *in vacuo*. After extraction with ether to remove the fat, the ether-insoluble residue is dried again. The yield is 3–5 g. per l. of milk. The enzyme keeps *in vacuo* for several weeks. It is used in the form of a 10% solution in 0·1 *M* phosphate buffer of *p*H 7·4.

15 mg. of the enzyme (0·15 ml. 10% solution) should oxidize 0·5 mg. hypoxanthine in less than 1 hr. (40°, *p*H 7·4). We discarded less active preparations as the O_2 uptake in the presence of such preparations tended to be (up to 15%) in excess of the theoretical value. It appears that enzyme preparations of low activity are obtained from milk contaminated with inhibitors [see Philpot, 1938].

The activity of the enzyme is tested manometrically. The following solutions are required:

(1) Hypoxanthine stock solution, 12·5 mg. in 50 ml. 0·025 *M* phosphate buffer of *p*H 7·4.

(2) Aqueous pigeon liver extract: 1 g. pigeon liver is thoroughly ground with sand and 10 parts of water and centrifuged. The supernatant liquid is used. It keeps for several weeks if stored in the refrigerator and preserved with octyl alcohol.

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(3) 10% xanthine oxidase in 0·1 *M* phosphate buffer, *p*H 7·4.
(4) Phosphate buffer, 0·1 *M*, *p*H 7·4.

In addition to the thermobarometer two conical manometric flasks with a centre chamber and a side arm are required. The first flask, serving as "blank" contains 0·5 ml. phosphate buffer, 3·5 ml. water and 0·5 ml. liver extract in the main compartment, 0·2 ml. 2 *N* sodium hydroxide in the centre chamber and 0·15 ml. xanthine oxidase solution in the side arm. The second flask contains 2 ml. hypoxanthine solution, 1·5 ml. water, 0·5 ml. phosphate buffer and 0·5 ml. liver extract in the main compartment. The centre chamber and side arm are filled as in the control. The bath temperature is 40°. The side arms are mixed when thermal equilibrium is reached and readings are taken at 10 min. intervals. Suitable enzyme preparations yield figures of the following order:

O_2 uptake after 10 min. 20 μl.
,, 20 min. 48 μl.
,, 30 min. 81 μl.
,, 40 min. 82·5 μl.
,, 60 min. 82·5 μl.
(Calculated: 82·4 μl.)

The O_2 uptake should be complete in less than 60 min. and should agree with the theoretical value within $\pm 2\%$.

Determination of uric acid

We used two methods for the determination of uric acid formed in the presence of xanthine oxidase: the method of Edson & Krebs [1936] based on the conversion of uric acid into urea, and Kern & Stransky's [1937] modification of the colorimetric method of Folin. The latter method, though less specific, is more convenient and is therefore preferable when substances interfering with the colour formation are absent. This was the case in our experiments and we have therefore used the colorimetric method in most determinations.

The method of Edson & Krebs can be employed without modification. If the colorimetric method is used the concentration of uric acid in the sample must be of the same order as in blood filtrates for which the method was originally designed. It is therefore essential to dilute the solutions if the concentration of uric acid is relatively high. The determination of the O_2 uptake in the presence of xanthine oxidase indicates the order of magnitude of the uric acid present and we have used this guide in the following way.

If the O_2 uptake is below 25 μl., the solution is rinsed with water into a 50 ml. measuring flask and water is added to fill about three-quarters of the flask. Then are added 1/100th volume of acetone (0·5 ml.), 1/25th vol. of 10% Na_2WO_4 (2 ml.) and 1/25th vol. of 0·66 *N* H_2SO_4. The flask is filled up to the mark and the contents filtered after 20 min. or more.

If the O_2 uptake is above 25 μl. the dilution is increased according to the following scale:

If the O_2 uptake is 25–50 μl. dilute to 100 ml.
,, 50–100 μl. dilute to 200 ml.
,, 100–200 μl. dilute to 400 ml., etc.

The relative proportions of acetone and deproteinizing reagents remain the same.

20 ml. filtrate are mixed with 5 ml. Na silicate solution (22 g. Na silicate cryst. Schering-Kahlbaum and 15 g. glycerol in 100 ml.) and 1 ml. Folin's phosphotungstic acid [see Kern & Stransky, 1937]. The colorimetric reading is taken after 5 min.

The standard uric acid solution is treated at the same time and in the same way as the unknown solutions: a known quantity of uric acid (about 0·03 mg.) is transferred into a 100 ml. flask and mixed with water and the reagents used in the xanthine oxidase experiments (xanthine oxidase, liver extract, buffer, acetone, tungstate, H_2SO_4) and filtered. 20 ml. of the filtrate are used.

Quantitative oxidation of purine bodies

Effect of liver extract. Hypoxanthine, xanthine and purine (see later) react with O_2 in the presence of xanthine oxidase as follows [Thurlow, 1925]:

$$-\underset{|}{N}=\underset{}{CH} + O_2 + H_2O \rightarrow -\underset{|}{N}=\underset{}{COH} + H_2O_2. \qquad \text{.....(1)}$$

The H_2O_2 formed inactivates and destroys the enzyme and unless it is removed reaction (1) does not go to completion. We found in accordance with Dixon [1925] and Dixon & Keilin [1936] that quantitative yields in respect to O_2 uptake and also to uric acid formation are obtained if pigeon liver extract, containing catalase, is added to xanthine oxidase. Mammalian liver is not suitable because it may contain uricase. Of the pigeon liver extract 0·5 ml. is added to 3–5 ml. solution; with smaller quantities we sometimes found incomplete uric acid yields though the O_2 uptake was correct.

Data on the O_2 uptake and uric acid formation in the presence of liver extract are given in Table I. The manometric arrangement was as described for the determination of xanthine oxidase activity.

Table I. *Oxidation of purine bodies in the presence of xanthine oxidase and liver extracts*

	O_2 consumption and uric acid formation				
	Quantity				
Substances added	mg.	In equivalents of μl. O_2*	O_2 uptake (μl.)	Uric acid formation (μl.)	Method used for uric acid determination
Hypoxanthine	0·918	151·2	150	150·5	Edson & Krebs [1936]
,,	0·612	100·8	101	99·5	,,
,,	0·306	50·4	49·3	52	,,
,,	0·153	25·2	25·7	25·8	,,
,,	1·028	169·0	174·5	172·5	Colorimetric
,,	0·514	84·5	87·0	83·5	,,
,,	0·257	42·3	42·5	43·0	,,
,,	0·129	21·1	18·0	21·7	,,
Xanthine	0·206	15·2	17·4	28·9	,,
,,	0·412	30·4	29·7	59·7	,,
,,	0·440	32·4	31·6	64·4	,,
Purine	0·476	132·5	133·5	86·2	,,

* Calculated on the assumption that hypoxanthine requires $1O_2$, xanthine $\frac{1}{2}O_2$ per mol.

Preparation of the solution for the determination of hypoxanthine and xanthine

The sample of purine base to be determined should be contained in a small volume of liquid (5–10 ml.) suitable for the manometric determination of the O_2 uptake. The reaction of the solution has to be adjusted with phosphate to

c. *p*H 7·4, but since the activity of xanthine oxidase does not vary much between *p*H 6·8 and 7·8, the adjustment need not be very accurate. The balanced salt solution of Krebs & Henseleit [1932] containing 0·025 *M* $NaHCO_3$ is adjusted to the correct *p*H by addition of 0·2 ml. 0·5 *M* KH_2PO_4 to 3 ml. saline.

If the purine bases are present in a volume too large for the manometric experiments, or if interfering substances (enzyme inhibitors) are present, it is advisable to precipitate the purines by means of the copper-bisulphite method and to redissolve them in a smaller volume. [See Krüger & Schmid, 1905; Thannhauser & Czoniczer, 1920; Kerr & Blish, 1932.]

Calculations

Let x_{O_2} = mol. O_2 absorbed in the presence of xanthine oxidase.
$x_{\text{Uric acid}}$ = mol. uric acid formed in the presence of xanthine oxidase.
$x_{\text{Hypoxanthine and xanthine}}$ = mol. hypoxanthine and xanthine present.

If hypoxanthine requires 1 mol. and xanthine 0·5 mol. O_2 for the conversion into uric acid (as is the case under suitable conditions):

$$x_{\text{Hypoxanthine}} = 2x_{O_2} - x_{\text{Uric acid}},$$
$$x_{\text{Xanthine}} = 2\,(x_{\text{Uric acid}} - x_{O_2}).$$

If there are other substances in addition to xanthine and hypoxanthine which absorb O_2 or produce uric acid in the presence of xanthine oxidase the formulae are not valid and the method is not applicable to the individual determination of hypoxanthine and xanthine. In this case, however, the method allows the calculation of the total purine bodies:

$$x_{\text{Total purine bodies}} = x_{\text{Uric acid}}.$$

Specificity of the method

Substances absorbing O_2 in the presence of xanthine oxidase. The specificity of xanthine oxidase, on which the method depends has recently been reviewed and examined by Booth [1938]. The enzyme absorbs O_2 in the presence of aldehydes and of a number of purine bodies. Among the purines Coombs [1927] found hypoxanthine, xanthine, 6:8-dihydroxypurine, 2-thioxanthine and adenine to react with the enzyme. Booth [1938] added 8-hydroxypurine and 2:8-dihydroxypurine to the list. Through the courtesy of Dr H. O. L. Fischer, Toronto, we were able to test another substance, purine, prepared by E. Fischer [1898]. We found that it reacts about as rapidly as hypoxanthine, yielding the theoretical amount of uric acid (Table II).

Table II. *Oxidation of purine in the presence of xanthine oxidase*

(Experimental conditions as described for testing the activity of xanthine oxidase; figures corrected for blanks.)

Substrate	0·476 mg. purine	0·514 mg. hypoxanthine	0·440 mg. xanthine
μl. O_2 absorbed after 20 min.	29·2	31·2	18
,, 40 min.	60·6	66·4	30
,, 60 min.	89·2	86·5	31·6
,, 120 min.	132·5	86·5	31·6
μl. O_2 calculated for oxidation to uric acid	133·5	85	32·4
mg. uric acid formed	0·646	0·615	0·483
mg. uric acid calc.	0·666	0·636	0·486

It follows that an O_2 uptake observed in the presence of xanthine oxidase does not finally prove the presence of xanthine or hypoxanthine. But of all the substances which may absorb O_2 in the presence of xanthine oxidase, only hypoxanthine, xanthine, adenine and a few aldehydes are known to occur in biological material. Uric acid determinations show whether aldehydes or purines are present: the presence of aldehydes is indicated if an absorption of O_2 but no formation of uric acid occurs. We have met with no such case in experiments on tissue extracts and body liquids, and we think it unlikely that aldehydes are found in animal tissues in quantities sufficient to interfere with the determination of purine bodies.

Adenine, if present, will be determined as hypoxanthine; its presence is suggested if the rate of oxidation is comparatively slow. Hypoxanthine reacts about 20 times faster than adenine [Booth, 1938].

Substances liberating hypoxanthine or xanthine. Nucleotides, nucleosides or guanine gradually liberate hypoxanthine or xanthine under the conditions of the determination. Among these potential precursors of purine bases muscle adenylic acid, free or as dinucleotide, is quantitatively the most important one in animal tissues. The method as described in this paper, does not distinguish between free and "bound" purine bodies (Table III). To differentiate between these, xanthine oxidase and catalase preparations free from nucleotidase, nucleosidase and guanase must be used. In the material, however, for which the method was designed, viz. pigeon liver, the concentration of bound purine is negligible as compared with the amount of free hypoxanthine produced and we have therefore made no attempts to prepare pure xanthine oxidase and catalase.

Table III. *O_2 uptake in the presence of adenylic acid and of guanine*

40 mg. "xanthine oxidase"; 0·5 ml. liver extract 1 : 10; 4 ml. *M*/40 phosphate buffer.

Substrate added:	4·25 mg. muscle adenylic acid	4·85 mg. yeast adenylic acid	2·1 mg. guanine	0·464 mg. hypoxanthine
μl. O_2 absorbed after 20 min.	6	2	6	20
,, 40 min.	16	5	11	72
,, 140 min.	63	27	26	77
,, 250 min.	91	42	38	77

Interfering substances

A number of substances interfere with the quantitative determination of the O_2 uptake because they undergo a "coupled oxidation" under the conditions of the determination. Such substances are nitrites [Thurlow, 1925] *p*-phenylenediamine [Harrison & Thurlow, 1926], methaemoglobin [Harrison & Thurlow, 1926; Dixon & Keilin, 1936], methyl, ethyl and propyl alcohols [Keilin & Hartree, 1936] and haemoglobin [Krebs, 1936; Bernheim *et al.* 1936]. Among these, haemoglobin and its derivatives are the only substances likely to be present in animal tissues. We find, however, that the traces of haemoglobin present in tissue extracts or in solutions in which slices have been kept do not affect the O_2 consumption if liver extract is present. Liver extract inhibits the coupled oxidation of haemoglobin and methaemoglobin (Table IV) although it promotes the coupled oxidation of alcohol [Keilin & Hartree, 1936]. If the concentration of haemoglobin or methaemoglobin is very high the inhibition of the coupled oxidation is not always complete. In this case, or in the presence

of alcohol, it is not possible to calculate the concentrations of hypoxanthine and xanthine from the O_2 uptake and only the concentration of the total purine bodies can be measured under these conditions.

Table IV. *Effect of liver extract on the "coupled oxidation" of haemoglobin*

Each flask contained 0·44 mg. hypoxanthine in 3 ml. 0·05 *M* phosphate buffer, *p*H 7·4; 15 mg. xanthine oxidase in 0·15 ml. The figures have been corrected for blanks. The haemoglobin solution was prepared by cytolysing washed pigeon red cells with 10 vol. of water. The solution was cleared by centrifuging. 1 ml. was equivalent to 108 μl. O_2.

Added to solution:		0·1 ml. haemoglobin solution	0·1 ml. haemoglobin solution; 0·5 ml. pigeon liver extract	0·5 ml. pigeon liver extract
μl. O_2 absorbed after 10 min.	28	48	22	24
,, 20 min.	46	98·5	66	66
,, 30 min.	56	128	70	71
,, 40 min.	64	130·5	71	74
,, 50 min.	66	132	72	74
Calc. for 1 mol. O_2	72·6	72·6	72·6	72·6

Summary

1. Conditions have been ascertained under which xanthine oxidase brings about the quantitative conversion of hypoxanthine and xanthine into uric acid.

2. A method based on the use of xanthine oxidase is described which permits the determination of hypoxanthine and xanthine in quantities from 0·1 mg. upwards. If both O_2 uptake and uric acid formation are measured it is possible to distinguish between hypoxanthine and xanthine, since the former requires 2 atoms, the latter 1 atom of oxygen.

3. In testing the specificity of the method it was found that purine reacts with xanthine oxidase as rapidly as hypoxanthine, but since purine has never been found in biological material it is improbable that it will interfere.

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CXXI. THE FORMATION OF HYPOXANTHINE IN PIGEON LIVER

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Edson *et al.* [1936] isolated hypoxanthine from solutions in which pigeon liver had been allowed to metabolize ammonium salts. Reindel & Schuler [1937] on the other hand, using alanine as substrate, found only traces of hypoxanthine and claimed that xanthine is the chief purine base synthesized in pigeon liver. In order to elucidate this discrepancy we re-examined the nature of the purine base formed using the method described in the preceding paper. In our former experiments hypoxanthine was isolated as hypoxanthine-$AgNO_3$ but the yields were not quantitative owing to unavoidable losses during the isolation and purification. The isolation experiments therefore left it open whether some xanthine is formed in addition to hypoxanthine. The new method enabled us to determine both purine bases quantitatively. The experiments confirm our previous results: hypoxanthine is virtually the only purine base synthesized in pigeon liver. Although the experimental conditions were widely varied we were unable to find significant quantities of xanthine.

The second part of the paper deals with the rate of hypoxanthine synthesis under varying conditions. Oxaloacetate and glutamine were found to increase specifically the rate of hypoxanthine synthesis. These substances may therefore be assumed to play a special part in the hypoxanthine synthesis, the nature of which, however, remains to be investigated.

Methods

The experiments were carried out in conical manometric flasks. Slices of pigeon liver were shaken at 40° in a saline medium containing 0·025 *M* $NaHCO_3$-CO_2 or phosphate buffer. Various substrates were added in the form of 0·2 *M* neutral solutions. After 1 or 2 hr. the slices were removed from the solution. If $NaHCO_3$-saline was used, 0·2 ml. 0·5 *M* KH_2PO_4 was added to the solution and 0·2 ml. 2*N* NaOH was placed in the centre cup of the manometric flask. The flask was then shaken for 20 min. in the water bath in order to remove the CO_2 from the solution. The flask was again disconnected; 1·0 ml. 0·1 *M* phosphate buffer of *p*H 7·4 and 0·5 ml. liver extract were placed in the main compartment and 0·2 ml. 10% xanthine oxidase in the side-arm. If the medium was phosphate saline the addition of KH_2PO_4 and the subsequent shaking for 20 min. were omitted. The O_2 uptake and uric acid formation were determined as described in the preceding paper. A blank determination on the reagents was carried out in all experiments.

Added hypoxanthine and xanthine were quantitatively recovered under these conditions.

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Nature of the purine base synthesized in pigeon liver

Some experiments in which the substrates and media were varied are recorded in Table I. The ratio $\frac{\text{uric acid}}{O_2}$ (last column) is, within the limits of error, 1·0, as is expected for hypoxanthine. The deviations are insignificant and in both directions. This leaves no doubt that the bulk of the purine base formed in pigeon liver is hypoxanthine.

Table I. *Determination of the purine base formed in pigeon liver*

40°. Period of incubation 60 min.

Substrate (final conc.)	Gas	Tissue mg.	In presence of xanthine oxidase: μl. O_2 absorbed	In presence of xanthine oxidase: μl. uric acid formed	Ratio $\frac{\text{uric acid}}{O_2}$
$NaHCO_3$ saline:					
—	5% CO_2 in O_2	27·7	24·9	24·0	0·96
l(+)Alanine (0·02 *M*)	,,	24·8	31·3	28·7	0·95
l(+)Glutamic acid (0·02 *M*)	,,	25·6	25·2	24·2	0·96
l(+)Glutamine (0·02 *M*)	,,	26·0	50·8	52·6	1·04
l(−)Aspartic acid (0·02 *M*)	,,	29·0	31·2	28·2	0·91
l(−)Asparagine (0·02 *M*)	,,	28·9	39·2	42·8	1·09
0·003 *M* NH_4Cl; 0·02 *M* pyruvate	,,	22·6	39·0	36·4	0·93
0·003 *M* NH_4Cl; 0·02 *M* oxaloacetate	,,	27·1	53·1	55·9	1·05
0·02 *M* Oxaloacetate; 0·02 *M* *l*(+) glutamine	,,	21·6	55·0	56·4	1·02
—	,,	14·06	15·0	13·3	0·89
—	5% CO_2 in N_2	44·04	12·5	11·2	0·90
0·04 *M* Pyruvate; 0·02 *M* NH_4Cl	5% CO_2 in O_2	17·04	36·1	33·2	0·91
0·04 *M* Oxaloacetate; 0·02 *M* NH_4Cl	,,	16·12	38·5	34·7	0·90
0·02 *M* Oxaloacetate; 0·01 *M* NH_4Cl	5% CO_2 in N_2	44·77	14·7	12·2	0·84
Phosphate saline:					
0·02 *M* Pyruvate; 0·01 *M* NH_4Cl	O_2	14·90	13·7	13·0	0·95
0·02 *M* Oxaloacetate; 0·01 *M* NH_4Cl	,,	9·57	23·5	22·8	0·97

Rate of hypoxanthine synthesis

Since hypoxanthine is the only purine base formed in pigeon liver, its concentration in the medium may be calculated directly from the amount of O_2 absorbed in the presence of xanthine oxidase. We have therefore dispensed with the determination of the uric acid formation in the further experiments which were carried out to measure the rate of hypoxanthine formation under varying conditions. Eleven sets of typical experiments are recorded in Table II. The rates are expressed by the quotient:

$$Q_{\text{hypoxanthine}} = \frac{\mu\text{l. } O_2 \text{ absorbed in the presence of xanthine oxidase}}{\text{mg. (dry) liver used} \times \text{hr. of incubation}}.$$

These experiments show the following:

(1) There is a small formation of hypoxanthine when no substrate is added (last column). This "blank" is still much smaller under anaerobic conditions (Exp. 4). It is probable that the hypoxanthine formed anaerobically does not arise from synthesis but from the breakdown of adenine nucleotides.

(2) Addition of amino-acid causes an increase of the rate of hypoxanthine synthesis (Exps. 1, 9). Among the substances tested glutamine has the largest effect (Exps. 1, 2, 3, 4, 5, 11). The increase does not occur under anaerobic conditions.

(3) NH_4Cl causes an increase of the rate of synthesis if added in low concentrations (0·005 *M*). In higher concentrations it tends to inhibit (Exp. 5).

Table II. *Rate of hypoxanthine synthesis in pigeon liver at 40°*

No.	Gas	Substrates (final conc.)	$Q_{hypoxanthine}$ With substrate	$Q_{hypoxanthine}$ No substrate
		$NaHCO_3$ saline		
1	5% CO_2 in O_2	0·02 *M* *l*(+)Alanine	1·26	0·90
	,,	0·02 *M* *l*(+)Glutamine	1·95	0·90
	,,	0·02 *M* *l*(+)Glutamate	0·99	0·90
	,,	0·02 *M* *l*(−)Aspartate	1·07	0·90
	,,	0·02 *M* *l*(−)Asparagine	1·35	0·90
	,,	0·02 *M* *l*(−)Arginine	0·81	0·90
	,,	0·0033 *M* NH_4Cl; 0·02 *M* oxaloacetate	1·97	0·90
	,,	0·02 *M* *l*(+)Glutamine; 0·02 *M* oxaloacetate	2·54	0·90
	,,	0·0033 *M* NH_4Cl; 0·02 *M* pyruvate	1·72	0·90
2	,,	0·01 *M* *l*(+)Glutamine	1·79	1·03
3	,,	0·01 *M* *l*(+)Glutamine	1·31	0·71
	,,	0·0066 *M* NH_4Cl; 0·02 *M* oxaloacetate	2·60	0·71
	,,	,, 0·02 *M* succinate	1·06	0·71
	,,	,, 0·02 *M* *l*(−)malate	0·82	0·71
	,,	,, 0·02 *M* acetoacetate	0·92	0·71
4	5% CO_2 in N_2	0·01 *M* *l*(+)Glutamine	0·28	0·30
	,,	0·0033 *M* NH_4Cl	0·26	0·30
	,,	0·0033 *M* NH_4Cl; 0·02 *M* pyruvate	0·31	0·30
	5% CO_2 in O_2	0·01 *M* Glutamine	1·08	0·89
	,,	0·0033 *M* NH_4Cl	0·64	0·89
	,,	0·0033 *M* NH_4Cl; 0·02 *M* pyruvate	1·20	0·89
5	,,	0·01 *M* *l*(+)Glutamine	1·01	0·36
	,,	0·02 *M* *l*(+)Glutamine	1·12	0·36
	,,	0·04 *M* *l*(+)Glutamine	1·21	0·36
	,,	0·005 *M* NH_4Cl	0·70	0·36
	,,	0·01 *M* NH_4Cl	0·53	0·36
	,,	0·02 *M* NH_4Cl	0·44	0·36
6	,,	0·01 *M* NH_4Cl	0·79	1·07
	,,	0·01 *M* NH_4Cl; 0·02 *M* pyruvate	2·12	1·07
	,,	0·01 *M* NH_4Cl; 0·02 *M* oxaloacetate	2·34	1·07
7	,,	0·01 *M* NH_4Cl; 0·02 *M* oxaloacetate	1·83	0·72
	,,	0·01 *M* NH_4Cl; 0·02 *M* oxaloacetate; 0·01 *M* *l*(+)alanine	1·72	0·72
	,,	0·01 *M* NH_4Cl; 0·02 *M* oxaloacetate; 0·01 *M* *l*(+)ornithine	2·11	0·72
	,,	,, 0·01 *M* *l*(+)arginine	1·94	0·72
	,,	,, 0·01 *M* *dl*-serine	1·49	0·72
	,,	,, 0·01 *M* *l*(−)asparagine	1·85	0·72
	,,	,, 0·01 *M* *l*(−)aspartate	1·61	0·72
		Phosphate saline		
8	O_2	0·01 *M* NH_4Cl	0·67	0·63
	,,	0·01 *M* NH_4Cl; 0·02 *M* pyruvate	0·92	0·63
	,,	0·01 *M* NH_4Cl; 0·02 *M* oxaloacetate	2·45	0·63
		$NaHCO_3$ saline		
9	5% CO_2 in O_2	0·01 *M* *l*(+)Ornithine	1·36	0·45
	,,	0·01 *M* *l*(−)Leucine	1·20	0·45
	,,	0·01 *M* *l*(+)Valine	0·99	0·45
	,,	0·02 *M* *dl*-Lysine	0·80	0·45
	,,	0·01 *M* *l*(−)Methionine	0·94	0·45
	,,	0·01 *M* *l*(−)Histidine	1·01	0·45
10	,,	0·01 *M* NH_4Cl; 0·02 *M* oxaloacetate	1·41	0·52
	,,	,, 0·02 *M* glucose	0·78	0·52
	,,	,, 0·5% glycogen	1·05	0·52
	,,	,, 0·02 *M* citrate	1·26	0·52
	,,	,, 0·02 *M* α-glycerophosphate	1·26	0·52
	,,	,, 0·02 *M* *l*(+)lactate	0·56	0·52
	,,	,, 0·02 *M* pyruvate	0·82	0·52
11	,,	,,	1·55	0·81
	,,	,, 0·02 *M* citrate	1·44	0·81
	,,	,, 0·02 *M* oxaloacetate	2·62	0·81
	,,	,, 0·02 *M* pyruvate	1·92	0·81
	,,	0·02 *M* *l*(+)Glutamine	1·67	0·81
	,,	0·02 *M* *l*(+)Glutamine; 0·01 *M* NH_4Cl; 0·02 *M* oxaloacetate	3·40	0·81
	,,	0·02 *M* *l*(+)Citrulline	0·92	0·81
	,,	0·02 *M* *l*(+)Citrulline; 0·01 *M* NH_4Cl; 0·02 *M* oxaloacetate	2·38	0·81

(4) Of the non-nitrogenous substances (when added together with NH_4Cl) oxaloacetate effects the greatest increase of the rate of hypoxanthine synthesis (Exps. 1, 3, 6, 8, 10).

(5) When glutamine, NH_4Cl and oxaloacetate are added together a summation of the effects sometimes occurs (Exp. 11).

(6) Amino-acids, when added together with NH_4Cl and oxaloacetate, have no significant effect on the hypoxanthine synthesis (Exps. 7, 11). There is thus no effect comparable with the action of ornithine on the urea synthesis in rat liver. This negative result does exclude the possibility that the substances tested play a part in the hypoxanthine synthesis, as a positive effect can only be expected if the concentration of the substance tested is a "limiting factor".

Hypoxanthine formation in other tissues

The livers of the fowl, duck, rat and guinea-pig were treated in the same way as pigeon liver, but no appreciable quantities of hypoxanthine were found when the conditions were aerobic. This may be expected since added hypoxanthine disappears in these tissues; they all contain xanthine oxidase. Anaerobically small quantities of hypoxanthine were liberated in these tissues, $Q_{\text{hypoxanthine}}$ being of the order 0·2.

Effects of glutamine and oxaloacetate on the uric acid synthesis in fowl liver

In view of the effects of oxaloacetate and glutamine on the hypoxanthine synthesis in pigeon liver we thought it of interest to investigate the effects of these substances on the corresponding reaction in fowl liver, the uric acid synthesis. Uric acid was determined colorimetrically, as described in the preceding paper. $Q_{\text{uric acid}}$, the rate of uric acid synthesis, is expressed in terms equivalent to $Q_{\text{hypoxanthine}}$. The results, recorded in Table III show that the corresponding effects do in fact occur.

Table III. *Effect of glutamine and oxaloacetate on the uric acid formation in fowl liver*

Substrates added (final concentration)	$Q_{\text{uric acid}}$
—	0·41
0·02 *M* Oxaloacetate; 0·01 *M* NH_4Cl	1·32
0·02 *M* *l*(+)Glutamine	1·33
0·02 *M* Pyruvate; 0·01 *M* NH_4Cl	1·04

Discussion

Reindel & Schuler's results. Although no explanation can be offered for the findings of Reindel & Schuler [1937] mentioned in the introduction, it appears certain from our experiments that xanthine is not formed *synthetically* in pigeon liver. It may be possible that traces of xanthine arise *hydrolytically* from guanine, or guanine nucleosides or nucleotides, since pigeon liver contains a guanase; but the quantities of guanine compounds present in the liver are almost negligible compared with the amounts of hypoxanthine synthesized in the presence of an excess of NH_4 salts.

The effect of oxaloacetate. The stimulating effect of oxaloacetate suggests the idea that oxaloacetate serves as the source of the carbon skeleton in the synthesis of the purine ring. Oxaloacetate, as an intermediate product in the oxidative

breakdown of carbohydrate, lactate and pyruvate belongs to the relatively small number of substances available in sufficient quantities.

The effect of glutamine. The facts at our disposal are not sufficient to explain the action of glutamine. It is improbable that the carbon chain of the glutamine molecule, or part of it, enters directly into the purine ring, but more likely that glutamine acts as an "ammonia carrier". It is of great interest in this connexion that Leuthardt [1938] found a somewhat analogous effect of glutamine on the urea synthesis in the liver of starved guinea-pigs.

These effects of glutamine, however, are not necessarily to be explained by a direct participation in the synthesis, since synthetic reactions are always complex processes connected with the production and transmission of energy. Several observations, such as the occurrence of a glutamine synthesis in brain cortex and in retina [Krebs, 1935] and the necessity of glutamine for the growth of *Streptococcus haemolyticus* [McIlwain *et al.* 1939], suggest that glutamine plays a more general, though still entirely undefined, role in cell metabolism.

Summary

1. The purine base synthesized by pigeon liver requires 1 mol. O_2 for its quantitative conversion into uric acid. The base is therefore hypoxanthine. Contrary results of Reindel & Schuler [1937] who claim to have found a formation of xanthine remain unexplained.

2. The effects of added substrates on the rate of hypoxanthine synthesis were studied. Glutamine and oxaloacetate were found to increase the rate of the synthesis. The cause of these effects is discussed.

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CXXII. THE SYNTHESIS OF GLUTAMINE IN PIGEON LIVER

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(*Received 1 May 1939*)

It has previously been shown [Edson *et al.* 1936] that pigeon liver utilizes added NH_3 more rapidly than can be accounted for by the rate of hypoxanthine synthesis. Under some conditions, for instance in the presence of 0·02 to 0·04 *M* ammonium pyruvate, the amount of NH_3 converted into hypoxanthine is less than 20% of total NH_3 metabolized; the bulk of the NH_3 is converted into substances which remained unidentified. We have now identified glutamine in this fraction. Its quantity accounts for about 40 to 50% of the total NH_3 metabolized.

Experimental

As in the previous experiments slices of pigeon liver suspended in the saline medium of Krebs & Henseleit [1932] were used. Small scale experiments were carried out in manometric flasks, large scale experiments in "metabolism flasks", as described by Krebs [1933]. These flasks have a diameter of about 11 cm. and they contained not more than 100 ml. medium in order to secure sufficient oxygenation. The temperature was 40°. Ammonia was determined according to Parnas & Heller [1924], amino-N according to Van Slyke [1929] after the removal of NH_3 from the solution.

Formation of "amino-nitrogen" in pigeon liver

Data concerning the rate of NH_3 uptake have already been given [Edson *et al.* 1936] and it has also been mentioned that part of the NH_3 is converted into amino compounds. In a series of further experiments we compared the rates of NH_3 uptake and of hypoxanthine and "amino-N" formation. The highest rates of NH_3 uptake and amino-N formation were observed in the presence of pyruvate. An example is given in Table I. In this experiment the amino-N formed accounts for most of the metabolized NH_3. "Amino-N", as determined by Van Slyke's method, it should be remembered, does not necessarily imply $=CHNH_2$ groups but would include among other groupings the $—CONH_2$ group of glutamine.

Table I. *Utilization of added NH_4Cl by pigeon liver*

The medium contained 0·04 *M* pyruvate and 0·01 *M* NH_4Cl; 44 mg. liver; 2 hr.; 40°.

mg. NH_3-N added	0·840
mg. NH_3-N used	0·795
mg. hypoxanthine-N formed	0·110
mg. "amino-N"	0·658

Data for control to which no NH_4Cl was added calculated for same quantity of tissue.

mg. NH_3-N formed	0·021
mg. hypoxanthine-N formed	0·081
mg. amino-N formed	0·163

[1] Rockefeller Travelling Research Fellow.

Formation of amide-nitrogen

If the solution from which NH_3 has been removed by pigeon liver is heated for 10 min. to 100° in the presence of 5% H_2SO_4, 15 to 25% of the NH_3 metabolized returns to the solution. Examples are given in Table II. Heating for

Table II. *NH_3-consumption and amide formation in pigeon liver*

No.	Substrate added (total volume of medium 3–4 ml.)	mg. tissue	Time min.	µl. NH_3 used	µl. amide-N formed	Q_{NH_3}	$Q_{amide\text{-}N}$
1	—	29·9	90	+ 26	39·6	+ 0·58	+0·88
	0·01 *M* NH_4Cl; 0·04 *M* pyruvate	29·6	90	− 792	131	−17·9	+2·95
	0·01 *M* NH_4Cl; 0·02 *M* *l*(+) glutamate	40·2	90	− 412	115	− 6·8	+1·91
	0·01 *M* NH_4Cl; 0·01 *M* α-ketoglutarate	36·0	90	− 447	113	− 8·3	+2·09
2	0·0066 *M* NH_4Cl	16·1	60	− 118	30	− 7·35	+1·87
	0·0066 *M* NH_4Cl; 0·02 *M* pyruvate	13·0	60	− 267	42·5	−20·5	+3·28
	0·0066 *M* NH_4Cl; 0·02 *M* oxaloacetate	10·9	60	− 186	31·7	−17·1	+2·92
3	0·01 *M* NH_4Cl	32·5	90	− 304	−76·5	− 6·25	+1·57
	0·01 *M* NH_4Cl; 0·02 *M* α-ketoglutarate	30·7	90	− 327	100	− 7·1	+2·17
	0·01 *M* NH_4Cl; 0·02 *M* *l*(+)glutamate	27·8	90	− 266	85·5	− 6·4	+2·05
	0·01 *M* NH_4Cl; 0·02 *M* pyruvate	28·7	90	− 618	145	−14·4	+3·37
4	0·02 *M* NH_4Cl	68·7	120	− 182	98	− 1·3	+0·71
	0·02 *M* NH_4Cl; 0·02 *M* pyruvate	73·2	120	−1070	198	− 7·3	+1·35
	0·02 *M* NH_4Cl; 0·02 *M* oxaloacetate	61·0	120	− 800	157	− 6·6	+1·28
5	0·02 *M* NH_4Cl	52·5	100	− 658	144	− 7·5	+1·6
	0·02 *M* NH_4Cl; 0·02 *M* pyruvate	38·4	100	−1090	257	−17·0	+4·0
	0·02 *M* NH_4Cl; 0·04 *M* pyruvate	37·3	100	−1122	313	−18·1	+5·0
	0·02 *M* NH_4Cl; 0·04 *M* *l*(+)lactate	47·6	100	− 419	146	− 5·4	+1·84
	0·02 *M* NH_4Cl; 0·04 *M* *l*(+)glutamate	42·0	100	− 589	248	− 8·4	+3·55
	0·02 *M* NH_4Cl; 0·04 *M* α-ketoglutarate	44·0	100	− 574	148	− 7·0	+2·28

more than 10 min. does not significantly increase the yield of NH_3. Alkaline hydrolysis (10 min., 100°, 2*N* NaOH) liberates the same quantity of NH_3 as acid hydrolysis. The solutions thus contain a compound which behaves like glutamine or similar acid amides [*vide* Krebs, 1935, 1].

The yield of amide-N is greatest in the presence of pyruvate. There is only a small increase over the blank when *l*(+)glutamate or α-ketoglutarate is added. In this respect pigeon liver behaves differently from a group of tissues comprizing mammalian brain and retina and the kidney of guinea-pig and rabbit in which a synthesis of glutamine was previously found to take place [Krebs, 1935]. In these tissues a rapid synthesis of glutamine occurs if ammonium glutamate is added. Ammonium pyruvate (and alanine) are also converted into glutamine but the rate of the synthesis is in this case no more than 10 or 20% of that observed in the presence of ammonium glutamate (Table III).

Table III. *Formation of glutamine in guinea-pig kidney*

40°; bicarbonate saline; 0·01 *M* NH_4Cl. 5% CO_2 in O_2.

Substrates (final concentration)	$Q_{amide\text{-}N}$
—	1·31
0·02 *M* pyruvate	2·40
0·02 *M* *l*(+)glutamate	12·9
0·02 *M* *l*(+)alanine	4·7
0·03 *M* α-ketoglutarate	3·6
0·02 *M* α-ketoglutarate; 0·02 *M* l(+) alanine	10·3

Isolation of the acid amide

The determination of amide-N on samples containing 0·05–0·1 mg. amide-N proved a convenient guide in the isolation of the amide. The amide compound is not precipitated by phosphotungstic acid, Cu or Ag salts, but forms an

insoluble compound with mercuric salts in weakly acid, neutral or weakly alkaline solution. After many preliminary tests the isolation was finally carried out in the following way.

Two or three livers of well fed, freshly killed pigeons are roughly weighed and then sliced with a razor. For each g. fresh weight 7 ml. saline [Krebs & Henseleit, 1932], 0·7 ml. 0·2 *M* NH_4Cl and 1·4 ml. 0·2 *M* sodium pyruvate are measured into a metabolism flask [Krebs, 1933]. The proportion of NH_4Cl to tissue chosen allows an almost complete utilization of the added NH_3 during an experimental period of 3 hr. The removal of NH_3 simplifies the purification of the substance.

The flasks are filled with 5% CO_2 in O_2 and shaken at 40° for 3 hr. at the rate of 40–60 periods per min. After shaking 0·2 vol. 30% trichloroacetic acid is added and the mixture is cooled in ice. It is filtered after about 30 min. A slight turbidity of the filtrate does not interfere with the later stages of the isolation and may be neglected. The filter is washed with 3% trichloroacetic acid.

The acid amide is then precipitated with the mercuric nitrate reagent introduced by Schulze [1882]. To each 50 ml. of the combined filtrate and washings are added 5 ml. of mercuric oxide, dissolved in nitric acid (160 ml. concentrated HNO_3, 300 ml. H_2O and 220 g. HgO are boiled under reflux until the oxide is dissolved. After cooling *N* NaOH is added to produce a faint opalescence. The volume is made up to 1 l. with H_2O).

A 30% NaOH solution is slowly added under constant shaking until the solution is distinctly alkaline (litmus). It is then placed in the refrigerator overnight. The precipitate is centrifuged and the supernatant is tested with a drop of NaOH. If a yellow precipitate is formed more NaOH is added. The precipitate which contains among other substances the mercury salts of the "amide" is washed twice on the centrifuge with distilled water. After the last washing the precipitate is suspended in water and treated with H_2S. The mercuric sulphide is filtered off and washed with water. The filtrate is concentrated *in vacuo* to about 2 or 3 ml., transferred into a centrifuge tube and dried in a vacuum desiccator.

The oily or dry residue is stirred with about 0·5 ml. water, 0·1 ml. conc. HCl and 3 ml. methyl alcohol; if an insoluble fraction remains, it is removed by centrifuging; the supernatant is mixed with sufficient ether to produce a turbidity. Crystals appear soon when the mixture is placed in the ice box. Addition of more ether increases the yield, but sometimes lowers the purity of the product. Yield: 30–50 mg. per 10 g. wet liver. The substance is recrystallized by dissolving it in a few drops of water, adding 4 vol. methyl alcohol and ether as described above.

The combined yields from several experiments were recrystallized until the melting point remained constant at 171–173° (uncorrected). The compound begins to darken a few degrees below this temperature and decomposes when melting. The analytical data (Dr Weiler and Dr Strauss) agree with those for glutamine hydrochloride ($C_5H_{11}N_2O_3Cl$):

C found:	32·91%; 33·05%;	calculated for $C_5H_{11}N_2O_3Cl$:	32·86%
H ,, :	5·68%; 6·13%;	,, :	6·07%
N ,, :	15·2%;	,, :	15·39%
Cl ,, :	18·8%;	,, :	19·4%

Treatment with chloramine T under the conditions described by Cohen [1939] for the determination of glutamic acid yielded succinic acid. From 2·87 mg. of

the substance 1·1 mg. succinic acid were obtained. The yield is not quantitative but of the same order as that obtained with pure glutamine (60–70%).

A preparation of glutamine hydrochloride obtained by dissolving glutamine from mangel-wurzels in a small volume of 10% HCl and precipitation with alcohol-ether was identical in every respect with the substance isolated from pigeon liver. The mixed melting points were unchanged.

The quantities of glutamine isolated in the pure state varied from 40 to 60% of the total "amide" present in the starting material. A considerable loss during the preparation is almost unavoidable owing to the instability of glutamine, and we therefore assume that glutamine represents most, if not all, of the amide synthesized in pigeon liver.

Glutamine synthesis in other species

Fowl and duck livers also form glutamine from added ammonium pyruvate and the rate was found to be about the same as in pigeon liver. Since the isolation of glutamine was carried out in the same way, these experiments are not described in full.

Rat liver, too, produces an acid amide, but in this case the rate is not appreciably increased over the blank when ammonium pyruvate is added. The maximum rate observed in rat liver was 1·0–1·5 ($Q_{\text{amide-N}}$), against values up to 5·0 in pigeon liver. The amide formed in rat liver has not yet been isolated and identified.

Discussion

Synthesis of glutamine from 3-carbon compounds. The synthesis of glutamine from ammonium pyruvate involves the formation of a 5-carbon chain. It is probable that the synthesis of α-ketoglutaric acid is the primary step in the formation of glutamine. Several mechanisms have previously been discussed for the synthesis of this compound [*vide* Krebs, 1935, 2; Weil-Malherbe, 1936; Krebs & Johnson, 1937] but it is yet too early to decide between the various possibilities.

Glutamine synthesis in guinea-pig kidney and bird's liver. It was pointed out that guinea-pig kidney (and some other tissues) synthesize glutamine more rapidly from ammonium glutamate than from ammonium pyruvate, whilst pigeon liver yields glutamine more rapidly when ammonium pyruvate is the substrate. Such differences in rates, in our view, do not necessarily indicate entirely different mechanisms; they may be explained on the assumption that the factors limiting the rate of glutamine synthesis are different in the two groups of tissues.

Significance of the glutamine synthesis. The significance of the synthesis of glutamine remains obscure as long as the role of glutamine in tissue metabolism is unknown. Some observations mentioned in the discussion of the preceding paper and the facts reported in this paper leave no doubt that glutamine is a factor of general importance in cell metabolism, but the nature of its function is not yet clear.

Summary

Slices of pigeon, fowl or duck liver form glutamine from ammonium pyruvate. The substance was isolated as glutamine hydrochloride; 1 g. of liver (dry weight) produces up to 30 mg. glutamine per hour.

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CXXIII. A METHOD FOR THE SEPARATION OF SUGARS BY THE CHROMATOGRAPHIC ADSORPTION OF THEIR COLOURED ESTERS

I. SEPARATION OF GLUCOSE AND FRUCTOSE

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In spite of the very large amount of work which has been done on the separation of individual carbohydrates from their mixtures the methods in general use are not very satisfactory if only small quantities of the substance are available. These methods depend ultimately on differences in solubilities or boiling-points of the carbohydrates or of their derivatives. The recent development of the method of chromatographic adsorption, has shown that the degree of affinity of adsorption may vary to a much greater extent with the molecular structure, than does the boiling-point or solubility. Further, the method of chromatographic adsorption can readily be adapted for microchemical work which is frequently essential in biochemical research.

From a large number of chromatographic tests carried out during the present investigation with sugars and their derivatives the best results were obtained with two groups of arylated derivatives, namely the azobenzene-*p*-benzenesulphonyl and azobenzene-*p*-benzoyl esters. This latter group has the advantage that the sugars are more easily separated by saponification after the chromatographic separation has been effected.

For brevity azobenzene-*p*-benzoyl chloride will be termed azoyl chloride, and the corresponding esters, azoyl esters.

The azoyl chloride reacts readily in pyridine solution with the hydroxyl groups of sugars. After reaction is complete the excess of azoyl chloride is converted into the methyl ester by the addition of methanol. The methyl ester can then be readily eliminated owing to its great solubility in most organic solvents.

The azoyl esters are coloured substances varying from orange to dark red. They are adsorbed from their solutions in organic solvents by many of the adsorbents commonly employed for chromatographic adsorption. Brockmann's Al_2O_3, treated with methanol for three days at 37° and dried in the air, possesses good adsorbent qualities for these esters but subsequent elution is not quantitative. Better results are obtained with silica (pure precip. B.D.H.) as the adsorbent. In this case the elution of the ester can easily be made quantitative.

The high molecular weight of the azoyl group and the fact that it is possible, in small adsorption columns, to carry out the separation of a few mg. of substances, makes the use of this method possible for microchemical work. For example, using a column 7 mm. in diameter and 150 mm. high it has been possible to carry out the separation of 10 mg. of glucopyranose ester and 10 mg. of fructopyranose ester which are equivalent to less than 2 mg. of each sugar. A preliminary account of this work has already been published [Reich, 1939].

Experimental

1. *Preparation of the azobenzene-*p*-benzoic acid*

This substance was prepared by Angeli & Valori [1913] by condensation of *p*-aminobenzoic acid and nitrosobenzene in glacial acetic acid. The authors give M.P. 241°. The following method has been found to give better yields. 69 g. of dry *p*-aminobenzoic acid are dissolved in 450 ml. of hot absolute alcohol and 80 ml. of glacial acetic acid are added to the hot solution. The solution is cooled to room temperature, 60 g. of thoroughly pulverized dry nitrosobenzene are added and the mixture is shaken until the latter has dissolved. The mixture is then kept for 60 hr. at room temperature, followed by 12 hr. at 0°. Most of the azobenzene-*p*-benzoic acid formed separates from the solution in crystals which are filtered off, well washed with water, dried *in vacuo* over $CaCl_2$ and recrystallized from boiling absolute alcohol, the concentrated solution being cooled slowly to 0°. The substance crystallizes as mats of red needles with a golden glint. A further portion of the pure substance can be obtained from the mother liquors by precipitation with water, and recrystallization from absolute alcohol. Yield about 70 % of the theoretical.

2. *Preparation of the azoyl chloride*

50 g. of the dry acid are mixed with 150 ml. of pure dry benzene. A solution of 40 g. of $SOCl_2$ in 100 ml. of benzene is then added and the mixture is heated under reflux for 1 hr. on a boiling water bath. The dark red solution is evaporated *in vacuo* and the dry residue redissolved in benzene. The benzene is again evaporated *in vacuo* and the operation repeated a third time. Finally the residue is dissolved in the minimum of boiling light petroleum (B.P. 60–80°), the hot solution is filtered through a heated Jena glass filter and the clear solution slowly cooled to 0°. Bright red crystals are obtained. Recrystallization may also be carried out from boiling benzene. In this case, beautiful dark red crystals with a purple metallic glint are obtained, M.P. 93°. The yield is quantitative. (Found: C, 64·04; H, 3·91; N, 11·8; Cl, 14·33 %. $C_{13}H_9ON_2Cl$ requires C, 63·8; H, 3·71; N, 11·5; Cl, 14·5 %.)

Recently Ladenburg *et al.* [1938] published a method for the preparation of azobenzene-*p*-benzoyl chloride. These authors state that they had difficulty in preparing the chloride from the acid, but were successful in preparing it by the action of a large excess of boiling thionyl chloride on a mixture of the acid with $2\frac{1}{2}$ times its weight of Na_2CO_3. They give M.P. 93–94° for the chloride. During the course of my work I have prepared several hundred g. of the chloride from the acid by the method described in the present paper and have always obtained a quantitative yield.

3. *Esterification of glucose; preparation of* α*-penta-azoyl-*d*-glucopyranose*

8·2 g. of well pulverized dry azoyl chloride are added to 50 ml. of pure pyridine (twice distilled over barium oxide) and cooled to −20°. The mixture is shaken for 30 min. at −20° and 1 g. of thoroughly pulverized dry glucose (6 hr. at 80° over P_2O_5 *in vacuo*) is added. The mixture is kept with frequent shaking for 4 days at 12°. It is then cooled to −20°, and 4 ml. of absolute methanol (dried over barium oxide and twice distilled), are added. The mixture is kept for 3 hr. at −20°, with frequent shaking and then for 12 hr. at 0°. The solid which separates is collected, dissolved in chloroform, precipitated with absolute ethanol, again collected and washed with absolute ethanol. The red powder thus

obtained is recrystallized from dioxane. Red crystals are obtained, M.P. 234–236°. Yield about 80 % of the theoretical. (Found: C, 69·32; H, 4·14; N, 11·36 %. $C_{71}H_{52}O_{11}N_{10}$ requires C, 69·8; H, 4·29; N, 11·47 %.) $[\alpha]_{4799}^{20°} = +193°$ (chloroform; $c = 1$).

4. *Esterification of fructose*

8·2 g. of well powdered azoyl chloride are added to 50 ml. of pure dry pyridine, previously cooled to −20°; the mixture is shaken for 30 min. at −20°, and 1 g. of well powdered dry fructose which had been dried in a very thin layer for 24 hr. over P_2O_5 *in vacuo* is added. The mixture is shaken for 4 hr. at −20°, and then left for 4 days at −12°, being frequently shaken during this time. It is then cooled to −20°, and 4 ml. of absolute methanol added. The mixture is kept for 3 hr. at −20° and then for 12 hr. at 0°. The solution is filtered and the filtrate evaporated *in vacuo* at room temperature in an apparatus designed by the author.[1] The residue is dissolved in the minimum of hot benzene and precipitated with absolute ethanol. The substance is recrystallized from CCl_4 and is obtained in the form of red crystals, M.P. 135–136°. (Found: C, 69·32; H, 4·47; N, 11·25 %. $C_{71}H_{52}O_{11}N_{10}$ requires C, 69·8; H, 4·29; N, 11·47 %.) $[\alpha]_{5459}^{20°} = -345°$ (chloroform; $c = 1$).

5. *Chromatographic separation of a mixture of azoyl esters of glucose and fructose*

(*a*) *Preparation of the adsorption column.* 70 g. of silica (pure precip. B.D.H.) are mixed with 300 ml. of a solution of 25 % benzene (pure dry) in dry light petroleum (B.P. 60–80°); the mixture is kept for about 30 min. The adsorption column is prepared with this mixture in the following way: a part of the mixture is poured into a tube 30 mm. in diameter and 400 mm. high. The bottom of the tube is closed by a wad of cotton-wool, on which is placed a perforated disc of porcelain. Establishing a pressure by compressed nitrogen from the top of the tube, the liquid is filtered through the column of silica, until only a few mm. of liquid remain above the surface of the silica. At this stage a fresh quantity of the mixture is poured into the tube and the filtration continued. This is repeated until the column acquires a height of about 300 mm. when a second perforated porcelain disc is placed at the top of the column, and about 100 ml. of the benzene-petroleum mixture (25 : 75) is filtered by pressure through the column, so that the latter is made compact and homogeneous before beginning the chromatographic adsorption. During this operation, as during the entire chromatographic adsorption, the column must never become dry. The pressure of the nitrogen is regulated during the formation of the column so that the liquid filters through the column at a speed of 6–10 ml. per min. A speed of 3–4 ml. per min. is used during the adsorption and development of the chromatogram.

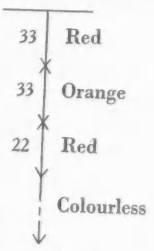

Fig. 1.

(*b*) *Adsorption.* In a typical experiment a solution of azoyl esters was prepared in the following way: 200 mg. of the glucose ester and 200 mg. of the fructose ester were dissolved in 40 ml. of chloroform, 80 ml. of benzene and 80 ml. of petroleum are added and the solution was filtered through the column. During the operation the formation of two red layers, separated by an orange layer was observed at the top of the column. When filtration of the solution of esters was nearly com-

[1] The apparatus is now obtainable through Gallenkamp & Co. Ltd.

plete, so that only a few mm. remained above the column, the chromatogram shown in Fig. 1 was observed. The numbers indicate the thickness of the layers in mm.

For the development of the chromatogram, 800–900 ml. of the benzene-petroleum mixture (25 : 75) were filtered through the column, the current of nitrogen being maintained at the end of the filtration until the column was completely dry. The distribution of the chromatogram at the end of the procedure is shown in Fig. 2.

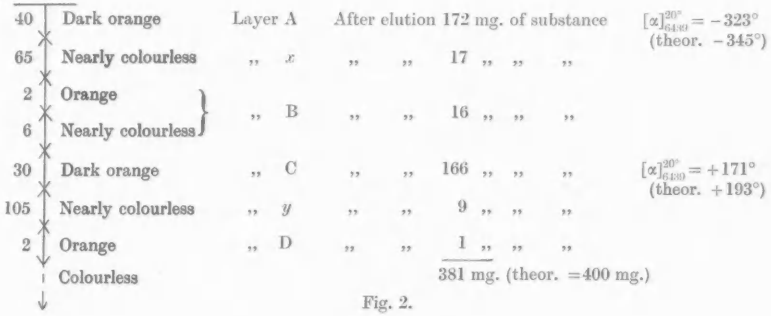

Fig. 2.

(*c*) *Elution.* For the separation of the different layers, the tube was held horizontally and, beginning with the upper layer, the column was scraped with a spatula, each layer being taken out separately and placed in a flask. For the elution of the adsorbed substance the powder was suspended in a mixture of methanol and chloroform (2 : 8), left for 12 hr. at room temperature and treated in the following way. Each mixture was filtered through a Jena glass filter No. G 3 (diameter 25 mm. height 150 mm.), a mixture of methanol and chloroform being drawn slowly through the powder on the filter until this became colourless. The clear filtrate was then evaporated *in vacuo*. The residue was dissolved in a small quantity of CCl_4, the solution filtered and the filtrate precipitated with light petroleum (B.P. 40–60°). After collecting the precipitate it was dried *in vacuo*. The yields of the substances eluted in this way are indicated in Fig. 2. One can see from the values of the specific rotation that, after a single adsorption, the substances are nearly pure. A second adsorption, carried out on the individual fractions, leads to the isolation of the pure substances.

Work upon the separation of other sugars and polysaccharides by similar methods is in progress.

Summary

1. By the action of azobenzene-*p*-benzoyl chloride (azoyl chloride) on glucose or fructose, in pyridine solution, coloured azoyl esters are obtained.

2. These esters can be adsorbed from their solution in organic solvents by various adsorbents from which they can be recovered by elution.

3. The adsorption is selective, allowing the separation of mixtures of these esters into their components by chromatographic adsorption.

4. The yields of pure substances, recovered from such mixtures are nearly quantitative.

5. The separation of a very small quantity (for example 20 mg.) of the sugar azoyl esters, can be executed by this method.

I wish to take this opportunity of thanking the Governing Body of the Lister Institute, for the hospitality of its laboratories and to Prof. R. Robison for the kindly interest he has shown in this investigation. I am indebted also to Dr Kenyon who placed at my disposal a cadmium lamp for polarimetric measurements.

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CXXIV. PARATHYROID HORMONE

I. ASSAY

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(*Received 13 April 1939*)

Collip's original method of assay of parathyroid hormone [Collip & Clark, 1925] presents certain serious disadvantages for routine laboratory use. Not only are dogs expensive and troublesome to maintain, but in practice it is impossible to obtain a sufficiently large group of animals of one breed. As a result, individual differences will be greater in a group of dogs than in an equally numerous group of inbred laboratory animals, such as the mouse and rat colonies available in every biological laboratory. Methods based on prevention of the toxic effects of injections of Na oxalate or $MgSO_4$ into mice or rats suffer from the disadvantage of requiring extremely numerous groups of animals, in order to give significant results [cf. Burn, 1937]; moreover, individual variations in susceptibility to both the toxic factor and the hormone tend still further to increase the variance. Dyer's rat urine method [1932; 1933; 1935] appeared to afford the most hopeful basis for the elaboration of a practicable and relatively trustworthy method of assay, an attempt at which is described in the present paper.

Experimental

Material and methods. The animal material consisted of male albino rats, of the State Hygiene Institute's inbred Wistar strain, weighing 150–200 g. each. Groups of 5 rats were placed in glass metabolism cages, of a type similar to that used by Dyer [cf. Burn, 1937], but with certain minor improvements. The cage (Fig. 1) consists of an inverted glass bell-jar, with a 0·5 cm. mesh Ni-plated brass wire netting floor and ceiling. The neck of the bell-jar is fitted with a thin glass rod, serving for suspension of a glass bulb, the drawn-out part of which dips into a 100 ml. Erlenmeyer flask, standing in a petri dish.

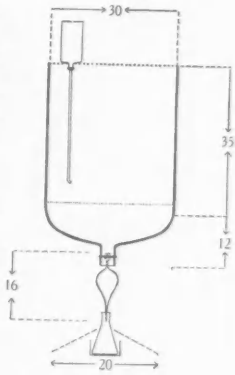

Fig. 1. Metabolism cage (to scale).

The dish is covered with a piece of fine-mesh wire gauze, in the form of a truncated cone. The cage, dish and outside of the flask are rinsed with small amounts of hot acetic acid once daily, the washings being added to the urine. The rats are fed once daily in a separate cage, the urine voided during this time being filtered immediately after feeding, and added to the main portion. Distilled water is allowed *ad lib.*

The diet consists of a mixture of wholemeal rye flour 70, evaporated skim milk 10, commercial casein 6, linseed cake 15, dried liver 3, $CaCO_3$ 0·5 and NaCl 0·5 parts, made into a stiff paste with 50 g. of butter and one raw egg per kg. of

mixture, together with the necessary amount of hot water. The mass is kneaded into ovoids, each weighing about 15 g., five of which are placed in the feeding cage. Feeding usually lasts about 1 hr., and scattering is practically absent. The combined urine + washings are diluted to 100 ml. in measuring flasks, and Ca is determined by the method of Truszkowski *et al.* [1938]. The values in all cases refer to urinary Ca output per 100 g. of rat per 24 hr.

Results

The appraisal of the potency of parathyroid preparations by a rat urine method requires that the standard deviation in urinary Ca output be determined for uninjected animals, in order to know what rise in urinary Ca might be regarded as being significant. Typical daily Ca excretion curves are given in Fig. 2,

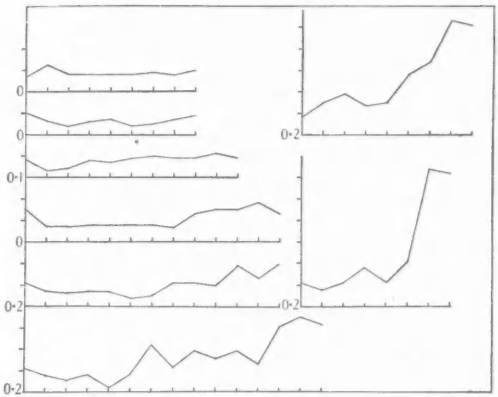

Fig. 2. Daily Ca excretion curves. Ordinates: Ca in 0·1 mg. Abscissae: days.

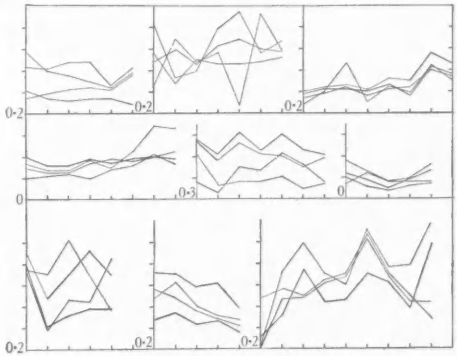

Fig. 3. Daily Ca excretion curves, for four groups studied simultaneously. Ordinates: Ca in 0·1 mg. Abscissae: days.

from which it appears that considerable variations in 24 hr. urinary Ca output may exist (see also Fig. 3), in spite of every precaution to avoid loss or contamination of urine, and of maintenance of standard feeding and living conditions.

Fig. 3 represents a number of groups of curves obtained for four groups of rats studied simultaneously. A general tendency towards parallel fluctuations is evident, suggesting that some factor responsible for variation in urinary Ca output affects all four groups simultaneously; the fluctuations are due not so much to individual group variability as to the action of some external, uncontrolled factor. As to the nature of this factor no basis for speculation is available. The phenomenon described may, however, be of practical importance for assay purposes, in as much as whilst the fluctuations for a given group of rats may be considerable, yet the differences between this group and a second control group may vary within narrower limits.

Fig. 4*a* represents the distribution of results of 792 determinations of urinary Ca (mg./100 g./24 hr.), under standard conditions, and Fig. 4*b* the same, for differences (283 values) between test and arbitrarily selected control groups. In

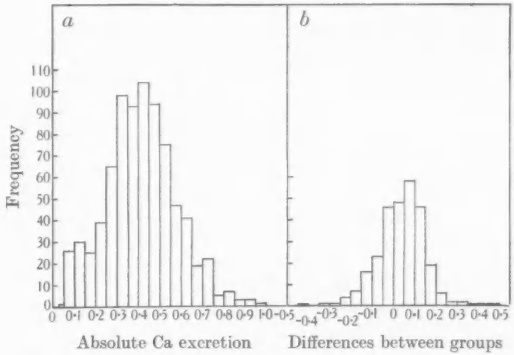

Fig. 4. Distribution of daily Ca excretion for groups of rats.

both cases the results correspond with a normal distribution. The standard deviation was calculated for both groups of results, from the equation

$$\sigma=\lambda\sqrt{\frac{\Sigma a^2 d}{n}-\left(\frac{\Sigma ad}{n}\right)^2-\frac{\lambda}{12}},$$

where λ is the class interval (0·05 mg.), a is the deviation, d the frequency and n the number of group-days. On this basis, Ca excretion, in mg./100 g. live wt./24 hr., was found to be 0·404, with $\sigma=0{\cdot}166$, whilst the difference between one group and another amounted to 0·064, with $\sigma=0{\cdot}066$. It follows that the standard deviation is 2·5 times as great for absolute Ca excretion as for the differences between groups. In other words, a rise or fall in Ca excretion equal to 0·166 mg./100 g./24 hr. could be expected once in 3 days, and in the difference between two groups on a given day once in 100 days. The conclusion is that a rise in the difference between two groups, of which one had received parathyroid hormone, is a more trustworthy indication of the activity of the hormone than is the absolute Ca rise in one group only.

Effect of injection of parathyroid hormone

Solutions of parathyroid hormone were prepared by a method based substantially on that of Tweedy [1930], from different batches of ox glands. Owing to technical difficulties, both in procuring large batches of glands, and in work-

ing them up in one operation, our preparations varied considerably in potency. Fig. 5*a* represents the effect of an injection, and Fig. 5*b* shows how the quantitative effect of this injection is evaluated. The curves of Fig. 5*a* represent daily variations in Ca excretion in two groups of rats. An injection of parathyroid hormone was given to one of the groups (curve 2) at the beginning of the 24-hr.

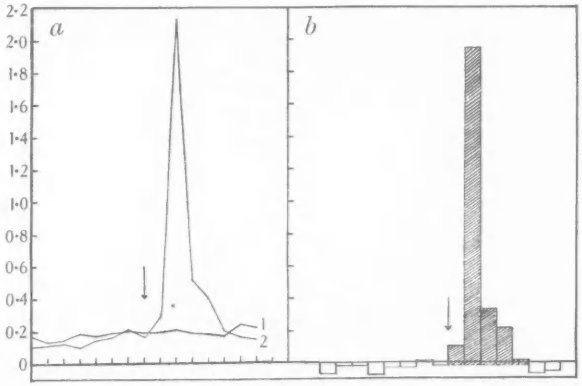

Fig. 5. Effect of injection of parathyroid hormone. Ordinates: Ca in 0·2 mg. Abscissae: days.

period marked by an arrow. The curve then rises and falls steeply, the effect being completed within 72 hr. The histogram (Fig. 5*b*) consists of a series of columns, each being the difference between the values of curves 1 and 2 on a given day; these differences may be positive or negative. The response to the injection is evaluated as being the sum of the areas of the columns (shaded) above the level of the pre-injection column. This procedure involves the arbitrary assumption that the level on the day preceding injection remains constant over the test period; actually, since the standard deviation is 0·066, it may vary considerably. The response is, however, considerably greater than these possible variations, which should not involve an error >10 %; such a margin of error is generally regarded as permissible in biological assay methods. The original histograms are drawn on millimetre square paper, to a scale of 1 cm./0·1 mg. Ca and per 24 hr. period, so that the sum S is expressed in sq. cm. We would propose that with this method of plotting 1 sq. cm. (=0·1 mg. Ca) be tentatively taken as being equivalent to 1 rat unit of parathyroid activity, pending the production and adoption of a reference standard. The potency of solid preparations of parathyroid hormone would then be best represented as $S/\log D$, where D is the dose in mg.

The groups of rats were discarded after each experiment, in view of the possibility of development of immunity in previously injected animals.

Uniformity of response of different groups of rats to an identical dose of hormone

An injection of 11·26 mg./100 g. of prep. A_c was given simultaneously to three groups of rats. The histograms representing the effect are shown in Fig. 6*a*. Fig. 6*b* illustrates the result of a similar experiment, taking 19 mg./100 g. of prep. Y. The values of S for prep. A_c at the given dosage level were $S_1=1·3$,

$S_2 = 2{\cdot}4$, $S_3 = 2{\cdot}3$; for prep. Y the values were $S_1 = 22{\cdot}6$, $S_2 = 25{\cdot}2$, $S_3 = 22{\cdot}9$ sq. cm. It follows that the responses differ considerably qualitatively, those to prep. A_c being distributed over 2, 3 and 1 days respectively for groups 1, 2 and 3, or

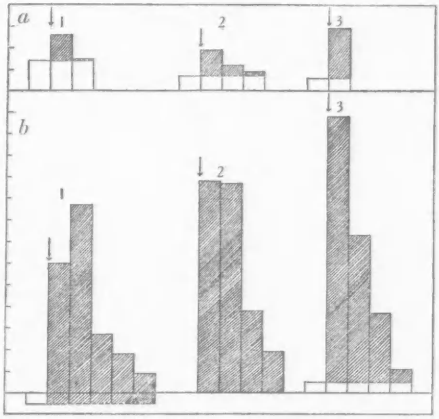

Fig. 6. Effect of equal doses of hormone prep. A_c (Fig. 6*a*) and Y (Fig. 6*b*) given to three groups of rats. Ordinates: Ca in 0·1 mg. Abscissae: days.

rising gradually to a low maximum for group 1 (prep. Y), and abruptly to higher ones in groups 2 and 3. The maximum level is maintained for 48 hr. in group 2, and for 24 hr. only in groups 1 and 3. Return to pre-injection levels is most gradual in group 3, less so in group 2 and least so in group 1. Yet the values of S differ relatively little for identical doses.

Gradation of response to various doses of a given preparation

It might be expected that the magnitude of S should bear some simple relation to the logarithm of the dose, were the assay method described to give a

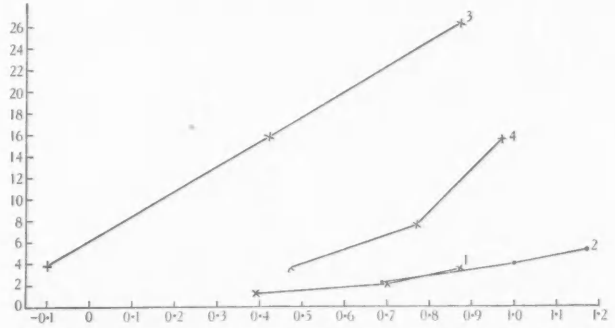

Fig. 7. Relation of dose of hormone to response. Ordinates: Response S. Abscissae: Logarithm of dose in mg.

true representation of the potency of the hormone preparations. The results obtained are shown in Fig. 7, in which curves 1 and 2 represent the effect of

injections of 2·5, 5·0 and 7·55, and of 5, 10 and 15 mg. of prep. N_2/100 g. of rat, made at different times into three groups of rats. Curve 2 is a straight line, and two of the points of curve 1 fall on the same line, the lowest dosage giving an aberrant effect. This is due probably to the magnification of the experimental error at this low dosage level. Curve 3 is a straight line joining the points obtained with doses of 0·78, 2·68 and 7·55 mg. of the more active prep. D. Curve 4 is for doses of 2·97, 5·95 and 8·87 mg. of prep. Z, and deviates to some extent from the rectilinear.

On the whole, the results confirm the validity of the assay method proposed.

Effects of multiple or single injections

It is known that for a number of hormones a better response is obtained when the dose is divided into a number of smaller ones, administered at intervals, and Dyer [1933] has reported the same for parathyroid hormone. A test made with one of our preparations showed that S was 20·1 when a given dose was administered in two injections on 1 day, and 26·0 when the same dose was spaced over 3 days. Whilst this result confirms Dyer's findings, yet the advantage does not in our opinion counterbalance the inconvenience of multiple injections, and of the prolongation of the test period. In subsequent work the hormone was administered in two injections at an interval of 4 hr.

Units

The present units are the Collip dog unit (one-hundredth of the amount of hormone causing a 5 mg. rise in dog serum Ca, after 15 hr.), or the Hanson unit, being one-hundredth of the amount of hormone raising the serum Ca of a parathyroidectomized dog by 1 mg. within 6 hr. These units are not very satisfactory, but no better ones have been proposed. Dyer bases his assays on comparison with the declared potency of a commercial preparation; such a procedure presents obvious disadvantages. The most satisfactory methods are those based on comparison with a standard preparation, but no such preparation is available in the present case. A sample of hormone was prepared in this laboratory about a year ago, and its stability is now being studied, with a view to the ultimate adoption of this or a similar preparation as a reference standard. In the meantime, the assay method proposed can be applied, if possible taking some reliable commercial preparation for comparison. In a test made in our laboratory it was found that a dose of 10 Collip units/100 g. of rat gave a response of $S = 19·3$, whence it would follow that 1 Collip unit is equivalent to about 2 of our rat units. The most potent preparation so far obtained in our laboratory had an activity of about 6 rat units/mg.

Summary

1. Fairly considerable fluctuations in urinary Ca output are established for groups of rats kept under standardized conditions.

2. A tendency towards parallel variation in Ca output of four different groups during given periods of time is evident; the standard deviation of differences between the various groups is 0·064 mg./100 g. live wt./day (283 values), as compared with 0·166 mg. for absolute urinary Ca output (792 values, of which the mean is 0·404 mg.).

3. The response to various doses of a given preparation of parathyroid hormone is proportional to the logarithm of the dose. For a given dose approximately the same response is given by different groups.

4. A graphical method of evaluating the potency of parathyroid preparations, based on that of Dyer, is described. Pending the establishment of a reference standard, it is proposed that activity be expressed in terms of excess of urinary Ca excretion following injection.

5. The proposed rat unit is defined as being one-tenth of the amount of hormone giving a total rise in urinary Ca output of 1 mg.

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CXXV. FORMIC DEHYDROGENASE OF *BACTERIUM COLI*: ITS INACTIVATION BY OXYGEN AND ITS PROTECTION IN THE BACTERIAL CELL

By ERNEST FREDERICK GALE

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(*Received 19 April 1939*)

QUASTEL & WHETHAM [1925] showed that washed suspensions of *Bact. coli* will reduce methylene blue in the presence of formate and that the rate of reduction under standard conditions is greater with formate as substrate than for any other substance, with the exception of the sugars. Stickland [1929] showed that if a suspension of the organism is incubated with *liquor pancreaticus* then the activity of the formic dehydrogenase with methylene blue increases until it reaches 4 or 5 times its original value after several days. The activity of other dehydrogenases also increases at first but then rapidly decreases until the preparation is eventually active only with formate. After filtration of this preparation through a glass filter, 50–60 % of the activity is found to reside in the filtrate which contains large quantities of cell debris. When these are removed by high speed centrifuging, the supernatant fluid is almost inactive. Although the original bacterial suspension is able to oxidize formate to completion, the dehydrogenase preparation is unable to react with O_2 in the presence of formate. A similar phenomenon was encountered by Stephenson [1928] with a preparation of lactic dehydrogenase from *Bact. coli* but, in that case, the preparation was able to utilize O_2 as H-acceptor in the presence of a trace of methylene blue as carrier. Stickland was unable to obtain an O_2 uptake with his preparation by the addition of 0·5 ml. methylene blue (1/5000) but obtained, with or without the dye, a small uptake amounting to 20–30 μl. of O_2. He was unable to find any explanation of this effect and could only obtain complete oxidation of formate by, first, the anaerobic reduction of a large quantity of methylene blue by the formate in the presence of the preparation, followed by the aerobic re-oxidation of the reduced methylene blue.

In the course of studies of the enzyme systems of *Bact. coli* by investigation of the material obtained by crushing cell suspensions in the wet-crushing mill of Booth & Green [1938] it was decided to reinvestigate this problem, with the results set out in the following paper.

Technique. The organisms in all cases were grown on the surface of broth-agar in Roux bottles for 24 hr. They were then washed off with distilled water, spun out, washed twice in water and finally suspended in distilled water. The strength of such suspensions was determined by means of a photoelectric turbidimeter [Clifton *et al.* 1935]. The Q_{O_2} (μl. O_2 taken up/hr./mg. dry weight of organism) was determined in Warburg manometers; 1 ml. each of the bacterial suspension, $M/40$ Na formate and $M/10$ phosphate buffer at pH 6·0 being placed in the main compartment of the manometer cup and 0·2 ml. 10 % NaOH in the centre cup. All values of Q_{O_2} are corrected for the blank respiration of the organism. The activity with methylene blue was determined in Thunberg tubes evacuated and incubated at 37°. The following quantities were used: 1 ml. bacterial suspension, 1 ml. $M/10$ Na formate (in the hollow stopper) and 1 ml.

phosphate buffer at *p*H 6·0, in the presence of 0·2 ml. of 0·5 % methylene blue. The substrate was tipped into the reaction mixture after equilibration and the time (T sec.) taken to reduce the dye to completion was noted. The activity of the dehydrogenase towards methylene blue is given as Q_{MB}, defined as: $Q_{MB} = \mu$l. O_2 equivalent to the methylene blue reduced/hr./mg. dry weight of preparation. Under the experimental conditions described, this is equal to $108{,}000/TW$, where W = dry weight of preparation.

For the preparation of cell-free material from the organisms, 30–40 Roux bottles were inoculated with the organism and incubated for 24 hr. The total quantity of cells so obtained was suspended, after washing, in 50 ml. of water and then ground for $2\frac{1}{2}$ hr. in the Booth-Green mill. The cream obtained in this manner was investigated for formic dehydrogenase activity as below.

Distribution of the formic dehydrogenase in the crushed material

The activity of the ground material as formic dehydrogenase was investigated by the methylene blue technique and the results compared with the activity of the original washed suspension of organisms. Next, the ground material (1) was centrifuged for 30 min. at 3000 r.p.m.; this treatment provides two fractions: a brownish-yellow opaque supernatant fluid (2) and a thick sediment (5). The material (2) was then further centrifuged at 11,000 r.p.m. for 20 min. and gave a clear yellow fluid (3). Finally, this clear fluid was filtered through a Seitz filter, giving a water-clear fluid (4). The dry weight of each fraction was determined by drying suitable amounts in tared vessels to a constant weight in a steam oven and the methylene blue activity (Q_{MB}) then determined as above. The results are set out in Table I.

Table I. *Distribution of formic dehydrogenase in crushed material*

	Q_{MB}	
Material	Exp. 1	Exp. 2
Washed suspension of organism	183	118
Ground material (1)	104	72
Opaque supernatant fluid (2)	38	25
Clear yellow fluid (3)	13	9
Filtered fluid (4)	0	0
Washed sediment (5)	218	136
Dried sediment	45	11

It is obvious from these results that the formic dehydrogenase activity resides in the solid particles, as fractional removal of these leads to a steady loss of activity, the final filtered fluid being inactive. Accordingly further work was carried out on the washed sediment (1) only. Attempts to remove the enzyme from the surface of the solid material by (i) extraction with phosphate buffers of *p*H 5–10 either before or after digestion with trypsin, (ii) digestion with trypsin, (iii) digestion with papain etc. were completely unsuccessful.

Oxidation of formate by the sediment. Reference to Table II shows that the sediment gives a Q_{O_2} of approximately the same value as that obtained with the whole organism but its Q_{MB} has decreased below that for the organism. The sediment incubated under the usual conditions with 1 ml. of $M/40$ formate gives a total oxygen uptake of 288 μl. so that the oxidation is taken to completion in agreement with the equation:

$$H.COOH + O = H_2O + CO_2 \text{ (theor. as above} = 280\,\mu\text{l.)}$$

This reaction has been established for the whole organism by Cook & Stephenson [1928].

Fig. 1 shows the effect of pH on the Q_{MB} and the Q_{O_2}. The former is more sensitive to changes in pH than the oxidation system. A working pH of 6·0 has been adopted for all experiments.

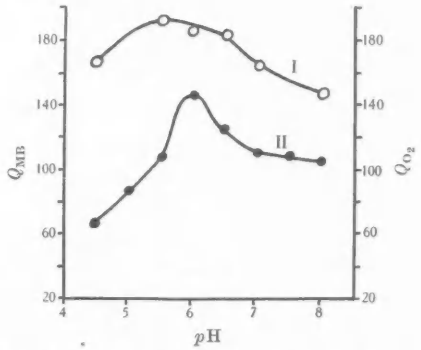

Fig. 1. Effect of pH on Q_{O_2} (I) and Q_{MB} (II) with formic dehydrogenase of the sediment.

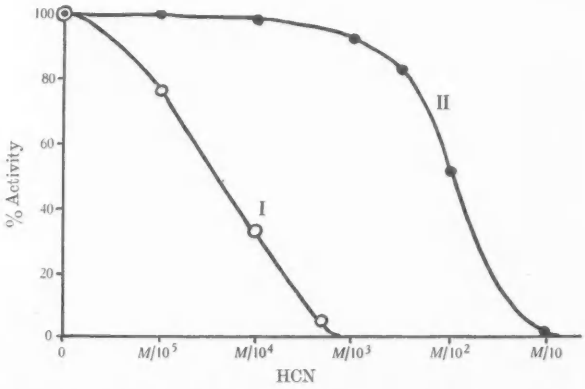

Fig. 2. Effect of HCN on Q_{O_2} (I) and Q_{MB} (II) of formic dehydrogenase in sediment.

Fig. 2 shows the effect of cyanide on the Q_{MB} and Q_{O_2}. The O_2 uptake is completely inhibited by a concentration of $M/1000$ cyanide but this concentration inhibits the methylene blue activity by 8 %, complete inhibition of the dehydrogenase only being obtained with $M/20$ cyanide. During the Q_{O_2} determinations mixtures of alkali and cyanide were used in the centre cup according to the technique of Krebs [1935]. From these results it appears that formic dehydrogenase differs from most dehydrogenases in being inhibited by cyanide. The preparation inactivated in the presence of $M/10$ cyanide is not restored by washing three times in 1000 times its weight of water so that the inhibition would appear to be irreversible. Adler & Svreenivasaya [1937] have also shown that formic dehydrogenase extracted from peas is inhibited by cyanide.

Effect of digestion of the sediment with trypsin. The sediment was next incubated in the presence of phosphate buffer at *p*H 7·5 with trypsin. 1 % fluoride was used as antiseptic as this has no effect upon the dehydrogenase activity. The trypsin preparation used was the "Pangestin" preparation of "Difco", in a concentration of 10 % of the weight of material to be digested. At intervals samples were withdrawn, washed thoroughly until free from trypsin, the dry weight determined and the Q_{MB} and Q_{O_2} investigated. Also the total O_2 uptake obtained in the presence of 1 ml. $M/40$ formate (theory = 280 μl.) with an amount of material equivalent to 2 mg. of the original sediment was determined. The results are given in Table II.

Table II. *Effect of digestion of sediment with trypsin*

Material	Digestion time	Q_{O_2} formate	Q_{MB}	Total O_2 uptake (μl.)
Washed suspension of organism	—	171	158	?
Washed sediment	—	166	107	290
	18 hr.	152	226	156
	40 hr.	132	366	102
	60 hr.	118	334	72
	6 days	114	603	34
	7 days	35	475	15
	8 days	—	442	0

From these figures it is seen that:

(1) The initial Q_{O_2} falls steadily but slowly for 6 days and then falls rapidly to zero by the 8th day.

(2) The Q_{MB} rises steadily until the 6th day and then falls but has 300–400 % of the original activity when $Q_{O_2} = 0$.

(3) The total O_2 consumption decreases rapidly as digestion proceeds.

During this digestion the dry weight of the sediment decreases to 25 % of its original value and the rise in Q_{MB} is largely accounted for by the decrease in dry weight due to removal of inactive material. However, in some cases, an increase in actual dehydrogenase activity amounting to some 200 % has been observed—as found by Stickland [1929]. Further digestion after the 8th day in the presence of fresh trypsin leads to a slow but steady loss in dehydrogenase activity.

Sediment allowed to autolyse in the presence of fluoride at 37° showed the changes set out in Table III. The experiments were all carried out with an amount of material equivalent to 2 mg. of the original sediment and the results show an increase in Q_{MB} while the Q_{O_2} remains unchanged but the total oxygen uptake is limited to 100–150 μl. after 6 days' autolysis.

Table III. *Effect of autolysis of sediment*

Material	Autolysis time	Q_{O_2} formate	Q_{MB}	Total O_2 uptake (μl.)
Washed suspension of organism	—	171	158	?
Washed sediment	—	166	107	290
	20 hr.	210	281	174
	40 hr.	184	281	154
	5 days	182	356	141
	6 days	153	324	137

It will be convenient to refer, in the following experiments, to the preparations as follows:

Material A. The untreated, washed sediment.

Material B. The sediment after 7 days' autolysis, able to oxidise 1 ml. $M/40$ formate to 50 % completion.

Material C. The sediment after 7 days' digestion, with a high Q_{MB} but unable to react with O_2.

In all cases a quantity of material, in suspension, was used equivalent to 2 mg. dry weight of material A. The materials were kept in water suspension in the presence of 1 % fluoride in the ice-chest; for use they were spun out, washed and resuspended in water. Desiccation of the materials leads to a marked loss of activity, see Table I.

It appears from the results obtained that the material C contains the formic dehydrogenase but that this is unable to react with O_2 in the absence of some factor which has been removed by the digestion with trypsin. This would also explain the differences between the methylene blue and O_2 activities in Figs. 1 and 2.

Spectroscopic examination of the materials. A sample of material A containing 50 mg./ml. was examined under the microspectroscope. No absorption bands were visible and slight warming produced no alteration. When hydrosulphite was added, the bands of cytochrome *b* were easily visible. On shaking the material with air the bands disappeared again. Next, formate was added to a fresh sample and, after momentary warming, the bands appeared strongly. The addition of $M/10{,}000$ cyanide had no effect on either the reduction of the cytochrome by formate or the reoxidation by shaking with air. $M/50$ cyanide inhibited the reduction of the cytochrome in the presence of the formate but not by hydrosulphite. This shows, from Fig. 2, that the formic dehydrogenase of the sediment can react with O_2 through the cytochrome system.

Examination of a similar sample of material B gave similar results except that the appearance of the cytochrome bands was slower in this case when the material was reduced with formate.

Material C, on the other hand, appeared to contain little or no cytochrome as no absorption bands appeared either on warming with formate or reduction with hydrosulphite. When a preparation of cytochrome *c*, extracted from heart muscle, was added to material C in the presence of formate and warmed, the spectrum of reduced cytochrome *c* appeared and the bands remained until a preparation of cytochrome oxidase from heart muscle was added, when the bands disappeared on shaking with air. Thus it would appear that the cytochrome *c*—cytochrome oxidase system can act as a carrier system in conjunction with the formic dehydrogenase of material C.

However, when material C was shaken in a Warburg manometer with phosphate buffer, formate and 0·2 ml. of a 0·06 % cytochrome *c* preparation and cytochrome oxidase, no significant O_2 uptake could be obtained. Further, material B, which contains cytochrome, is unable to oxidize 1 ml. of $M/40$ formate to completion but gives an O_2 rate which falls off steadily to zero after about an hour. The effect appears to be due to inactivation of the enzyme system and this was the next effect studied.

Inactivation of material B. Fig. 3 shows the oxygen consumption under standard conditions with varying initial concentrations of formate and 1 mg. (dry weight) of material B. Table IV shows the percentage theoretical uptake obtained in each case together with the initial Q_{O_2} determined for the first 10 min. of the reaction. The higher the initial formate concentration, the higher the initial Q_{O_2} but the lower the percentage oxidation of substrate.

Stickland [1929] stated that the possible by-products of the dehydrogenation, oxalic acid and formaldehyde, do not affect the reaction until their concentration

reaches a higher level than could be formed from the formate present. This was confirmed, $M/100$ oxalate or $M/100$ H.CHO having no effect.

Particular attention was paid to the possibility of the formation of H_2O_2 and this was ruled out from the following considerations:[1]

(1) All the preparations contain powerful catalase activity.

(2) The addition of alcohol in an attempt to obtain a coupled oxidation in the presence of catalase [Keilin & Hartree, 1936] has no effect on the O_2 consumption. Further, tests for acetaldehyde after such experiments proved negative. The test used was the fuchsin sulphurous acid spot test which, according to Feigl [1937], will detect $4\mu g.$ of acetaldehyde.

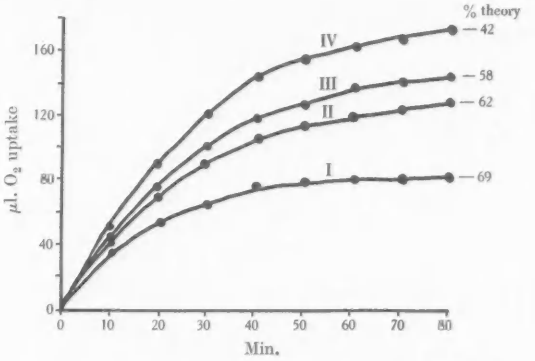

Fig. 3. Effect of initial formate concentration on inactivation, see Table IV. Initial concentration: I, $M/240$ formate. II, $M/160$ formate. III, $M/120$ formate. IV, $M/80$ formate.

Table IV. *Effect of initial formate concentration on inactivation*

ml. of $M/40$ formate added	Theoretical O_2 uptake $\mu l.$	Actual O_2 uptake $\mu l.$	% uptake	Initial Q_{O_2}
0·5	140	96	69	142
0·75	210	131	62	148
1·0	280	165	58	208
0·75 ($M/20$)	420	175	42	279

(3) No colour can be obtained, after a reaction, with guaiacum and peroxidase.

(4) $M/3000$ H_2O_2, easily detectable by the above test, has no effect on the Q_{O_2} or on the total O_2 consumption.

(5) The presence of peroxidase and *p*-phenylenediamine during the reaction has no effect on the Q_{O_2} or on the total O_2 consumption.

(6) In (5) slight browning of the reaction mixture occurred during the experiment but there was no difference in the depth or rate of appearance of the colour in the experiment and in the control without formate.

In case the effect should be due to a loss or deficiency of some factor in the oxidation carrier system, the addition of the following substances was tested: cozymase, lactoflavin, cytochrome oxidase, cytochrome *c* (0·2 ml. of 0·06 % solution, see p. 1023), boiled organism, malic and fumaric acids. The inactivation was not altered or checked by any of these additions or by combinations of these substances.

[1] See also Bhagvat & Hill (in preparation).

Conditions for inactivation. Fig. 4 shows the O_2 uptakes in two manometers containing material B in the usual amount. In manometer 1 (curve 1) 0·5 ml. of $M/40$ formate was tipped into the reaction mixture at zero time and the reaction allowed to proceed until the preparation was inactivated after 70 min. Then a further 0·5 ml. of $M/40$ formate was tipped in: the preparation was then unable to oxidize the freshly added substrate. In manometer 2 (curve 2) no formate was added at first but the enzyme preparation was shaken with buffer in air under similar conditions to those in manometer 1. Then 0·5 ml. of $M/40$ formate was added at the same time as the second addition to manometer 1. In this case the enzyme was able to oxidize the formate readily.

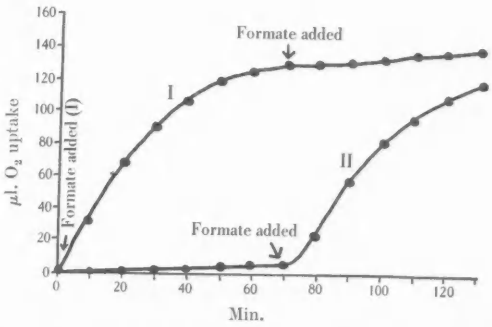

Fig. 4. Effect of presence of substrate on inactivation, see text.

This experiment shows that the enzyme is not inactivated by shaking in air under the experimental conditions but that the presence of the substrate is essential for the inactivation to occur. It also disposes of the supposition that the falling off in the rate of oxidation might be due to the establishment of an equilibrium since, in manometer 1, the addition of further reactant has no effect upon the course of the reaction.

In this experiment, the Q_{MB} of the preparation at the beginning of the experiment was 475. After shaking in air alone at 37° for 70 min., the value of the Q_{MB} was unchanged but the enzyme which had been incubated aerobically with the formate for the same time was completely inactivated. Thus the inactivation applies not only to the O_2 uptake but also to the methylene blue activity so that it is the dehydrogenase itself which is inactivated.

In order to determine whether the enzyme would be inactivated anaerobically in the presence of its substrate, 1 ml. of preparation containing 1 mg. dry weight of material B was incubated in a Thunberg tube with 1 ml. of $M/20$ formate and 1 ml. of phosphate buffer at pH 6; 2 ml. of 0·5 % methylene blue were tipped in slowly from the hollow stopper as the reduction progressed in the evacuated tube. The whole of the dye was reduced, being equivalent to an O_2 consumption of 300 μl., and the Q_{MB} of the material was unchanged. A similar experiment carried out aerobically in the absence of methylene blue gave an oxygen consumption of 113 μl., after which the enzyme was completely inactivated, having a Q_{MB} of less than 10.

Thus the dehydrogenase is inactivated only if it is incubated aerobically in the presence of its substrate.

Effect of incubation with glutathione (GSSG). Hopkins & Morgan [1938] have shown that the activity of succinic dehydrogenase of animal tissues is completely abolished by incubation with GSSG. The inactive material can then be reactivated by incubation with GSH and the authors suggest that the activity of the enzyme may depend upon the presence of certain SH groups. In order to test whether any such effect could be obtained with the formic dehydrogenase preparation, the following experiment was carried out: a series of Thunberg tubes were made up as set out in Table V and then incubated anaerobically for the times indicated. After this period the material was centrifuged out, washed, resuspended in water and the reduction time determined as usual in each case. It is seen from Table V that GSSG has no effect on the activity in the absence of formate but produces a slow decrease of activity when incubated with the dehydrogenase in the presence of its substrate.

Table V. *Effect of GSSG on formic dehydrogenase*

	Preliminary incubation. ml. of additions					
Tubes	1	2	3	3*a*	4	4*a*
Material B (2 mg./ml.)	2	2	2	2	2	2
M/20 phosphate	2	2	2	2	2	2
M/10 formate	—	1	—	—	1	1
M/50 GSSG	—	—	1	—	1	—
M/10 GSSG	—	—	—	1	—	1
Water	2	1	1	1	—	—

	Activity after incubation	
	Reduction times after	
Material from tube no.	2 hr. incubation min. sec.	1 hr. incubation min. sec.
1	7 0	6 30
2	7 10	6 30
3	7 5	6 30
4	8 5	8 30
3*a*	—	6 30
4*a*	—	10 30

Reactivation of inactivated enzyme. 10 ml. of a suspension of material B were incubated in the presence of 10 ml. *M*/10 formate and 10 ml. *M*/20 phosphate buffer at *p*H 6 for 2 hr., being bubbled vigorously with air throughout the incubation period. At the end of that time, a sample was taken and its Q_{MB} determined. The remainder of the material was centrifuged out, washed once and made up to 9 ml. with water. 1 ml. was placed in each of eight Thunberg tubes and the various additions indicated in Table VI then made. The tubes were incubated, 1 and 3 aerobically and the rest anaerobically, for 1 hr. In the hollow stoppers 1 ml. *M*/10 formate and 0·2 ml. 0·5 % methylene blue (the latter omitted in tubes 3 and 4) were placed and after the preliminary incubation, the contents of the stoppers were tipped in, all the tubes evacuated and the Q_{MB} determined in each case. From Table VI it is seen that anaerobic conditions in the presence or absence of formate succeed in reactivating the enzyme. GSH has no significant effect.

Fig. 5 shows the results of an experiment in which the rate of reactivation of a partially inactivated preparation was studied under anaerobic conditions and in the presence of formate anaerobically, by means of an experiment similar to the last in which the contents of the stoppers were added at 30 min. intervals. The presence of the formate accelerates the reactivation process. Since the inactivation process occurs only in the presence of formate, it is possible that the

Table VI. *Reactivation of inactivated dehydrogenase*

Tube	Conditions during preliminary incubation Additions	Q_{MB} after treatment
	Original material ...	442
	After aerobic incubation with formate	43
1	1 ml. water (aerobic)	43
2	1 ml. water (anaerobic)	366
3	1 ml. water 0·2 ml. 0·5 % MB (aerobic)	43
4	1 ml. water 0·2 ml. 0·5 % MB (anaerobic)	345
5	1 ml. *M*/100 GSH (anaerobic)	172
6	1 ml. *M*/100 GSSG (anaerobic)	108
7	1 ml. boiled organism (anaerobic)	21
8	1 ml. *M*/10 formate (anaerobic)	377

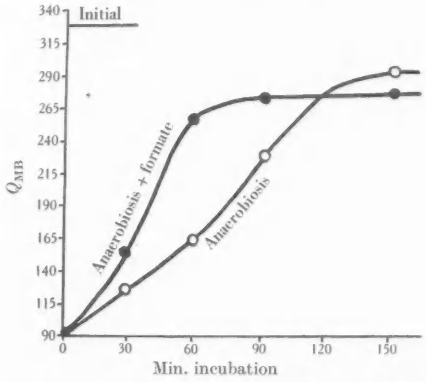

Fig. 5. Reaction of partially-inactivated enzyme by incubating, (*a*) anaerobically; (*b*) anaerobically with formate.

effect of anaerobic conditions alone in this last result may be due to the presence of unremoved traces of formate. It has not been possible to establish this as prolonged washing of the inactivated material renders it permanently inactive.

It would seem likely that the reactivation process is one of reduction. Moreover, the conditions necessary for the inactivation of the enzyme in the first place make it appear that the inactivation is associated with an oxidation of the dehydrogenase during its action with its substrate. Reference to Table II shows that the enzyme becomes more susceptible to the inactivation process as digestion of the sediment progresses. Further, it can be shown that the cytochrome content of the sediment decreases during digestion until material C is devoid of cytochrome. The question then arises as to whether the cytochrome normally "protects" the enzyme from inactivation by oxidation and this point will now be considered.

Effect of O_2 tension on the inactivation of material B

A series of manometers were set up with the usual contents for the investigation of the Q_{O_2} of material B. These were then gassed with O_2-N_2 mixtures containing the following percentages of O_2: 100, 50, 20, 10, 5, $2\frac{1}{2}$. Fig. 6 shows the O_2

uptakes followed over 10 min. intervals. In pure oxygen, the enzyme is quickly inactivated, the oxygen uptake being reduced to zero in 30 min. As the percentage of O_2 in the gas phase decreases so does the rate of inactivation until, in $2\frac{1}{2}$ % O_2, the O_2 consumption is linear for at least 2 hr.

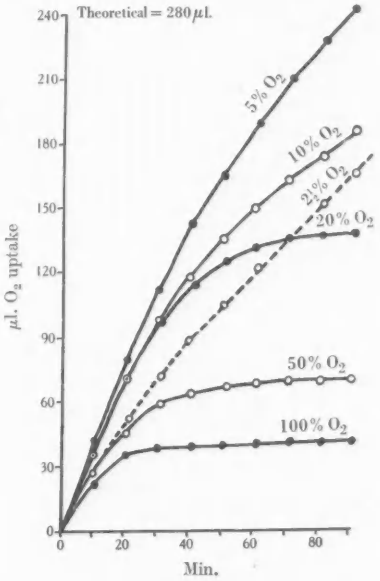

Fig. 6.

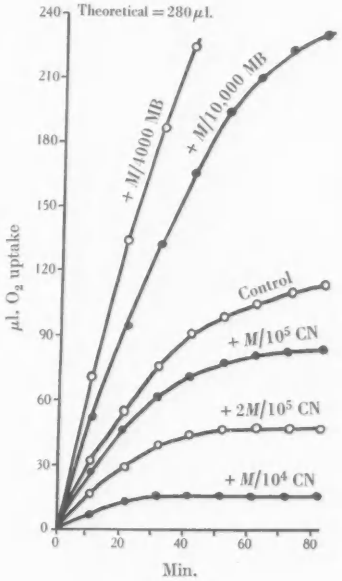

Fig. 7.

Fig. 6. Effect of oxygen tension on inactivation of formic dehydrogenase in material B.

Fig. 7. Effect of (*a*) MB; (*b*) CN on inactivation of formic dehydrogenase of material B in air.

Next the effect of altering the amount of potential oxygen-carriers in the system was tried. The rate of reaction of the dehydrogenase with O_2 through carrier systems can be increased by the addition of methylene blue, or decreased, if the natural carrier present is cytochrome as indicated by the spectroscopic observations, by the action of cyanide. Fig. 7 shows the effect of adding methylene blue to final concentrations of $M/4200$ and $M/10{,}500$ and of cyanide to $M/10^5$, $2M/10^5$ and $M/10^4$, the gas phase in all cases being air. Increasing the velocity of the reaction of the dehydrogenase with O_2 through carrier systems results in an inhibition of the inactivation process; while the action of cyanide, which inhibits the reaction through the cytochrome system, speeds up the inactivation of the dehydrogenase.

These effects can be reconstructed using methylene blue as the sole carrier with material C which contains no natural carrier. The material C when shaken with formate aerobically gives an O_2 uptake of 5–12 µl. and is then inactivated towards either O_2 or methylene blue. Fig. 8 shows the effect of adding various concentrations of MB to material C and then shaking with formate in air. Low concentrations of the dye—less than $M/10^5$—have very little effect but higher concentrations enable O_2 to be utilized to an extent depending upon the

dye concentration: the higher this concentration, the slower the inactivation of the dehydrogenase.

Fig. 9 shows the course of the O_2 uptake by material C in the presence of formate and a constant amount of MB, $M/4200$, but with various O_2 tensions. High percentages of O_2 in the gas phase lead to a rapid inactivation of the dehydrogenase while if the O_2 tension is sufficiently reduced, then a linear uptake is obtained over the experimental period. The general picture is thus similar to that of Fig. 6 in which the carrier system is cytochrome.

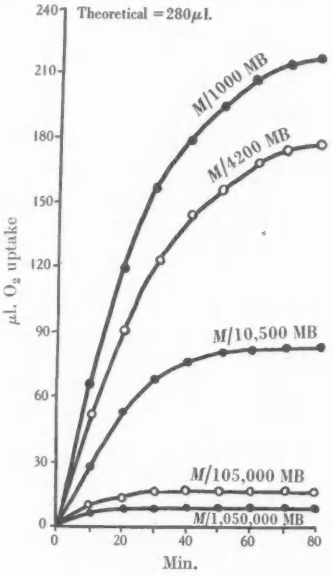

Fig. 8.

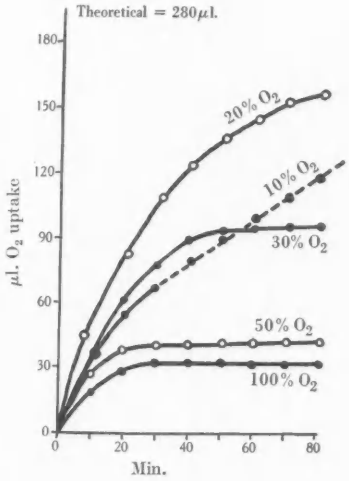

Fig. 9.

Fig. 8. Oxygen uptake with material C in presence of formate and varying concentrations of MB in air.

Fig. 9. Course of O_2 uptake with material C in presence of formate, $M/4200$ methylene blue and varying oxygen tensions.

In every case, the rate of inactivation of the dehydrogenase is accelerated by decreasing the concentration of the oxygen-carrying systems present, but is inhibited by increasing their concentration. In the presence of a constant amount of carrier, then the rate of inactivation is dependent upon the O_2 tension; the higher the tension, the more rapid the inactivation process.

It would appear that the enzyme can react with O_2 along two paths—directly, leading to inactivation of the dehydrogenase activity, and indirectly through a carrier system; which process predominates depends upon (*a*) the O_2 tension and (*b*) the amount of carrier present. Increasing the O_2 tension, while the amount of carrier present is unchanged, would speed up the direct reaction and thus the inactivation process; while increasing the amount of carrier favours the indirect reaction and so diverts the O_2 from the inactivation process. Under physiological conditions it is probable that the reaction through the natural

carrier system is so rapid that the O_2 is diverted from the direct reaction and inactivation proceeds only slowly. This is dealt with later.

Reconstruction of the cytochrome system. Previously it has been impossible to obtain an O_2 uptake by material C in the presence of formate by the addition of cytochrome *c* and cytochrome oxidase. It would appear that this result may be due to (*a*) insufficiently high concentration of cytochrome and (*b*) too high O_2 tension. Also it must be remembered that cytochrome *c* is not the natural cytochrome involved but no method of preparing the cytochrome *b* of bacteria is yet available. Hence manometers were set up with the following additions:

(1) and (3) 0·5 ml. 1 % cytochrome *c*; 0·3 ml. cytochrome oxidase.

(2) 0·25 ml. 1 % cytochrome *c*; 0·2 ml. cytochrome oxidase.

Manometers 1 and 2 were filled with O_2-N_2 mixtures containing 4 % O_2 and manometer 3 was filled with air. On incubation with formate, and material C, manometer 3 gave an uptake of 10 μl. in 30 min. after which the enzyme was inactive. Manometer 1 showed a linear O_2 uptake lasting for 2½ hr. before the experiment was stopped: the steady $Q_{O_2} = 29$. Manometer 2 also showed a small O_2 uptake ($Q_{O_2} = 13$) which decreased slowly after 90 min. None of the controls from which any of the components (enzyme-formate-cytochrome-cytochrome oxidase-oxygen) was missing showed any significant activity. (I am indebted to Dr Hartree of the Molteno Institute, Cambridge, for the concentrated solution of cytochrome *c* and the cytochrome oxidase preparations.)

This reconstruction proves that the formic dehydrogenase of *Bact. coli* reacts with O_2 through the cytochrome system.

Effect of oxygen on the formic dehydrogenase of Bact. coli *grown in air.* A culture of *Bact. coli* was grown on the surface of broth agar in air for 20 hr., washed twice and resuspended in distilled water; dry weight of suspension = 1·2 mg./ml. Manometers were then set up containing 1 ml. bacterial suspension, 1 ml. phosphate buffer *p*H 6 and 1 ml. *M*/40 formate (water in controls); the manometers were filled with O_2-N_2 mixtures containing air, 30, 50, 100 % O_2 respectively. The course of the O_2 uptake at 37° under these conditions is set out in Fig. 10. An oxygen tension greater than that in air has an inactivation effect depending upon its tension. In order to investigate this further, two terms are defined:

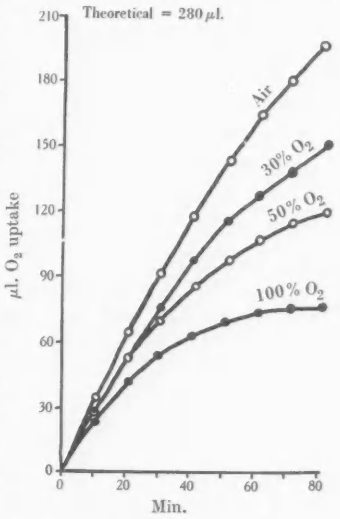

Fig. 10. Oxidation of formate by *Bact. coli* grown in air—effect of O_2 tension on oxygen uptake.

Critical oxygen tension: the maximum O_2 tension at which the rate of O_2 consumption is not more than halved in 1 hr.

Protection index: the number of minutes in which the O_2 consumption is halved when the system is acting in 100 % O_2.

For the organism grown in air, the critical O_2 tension is that holding in air, 21 %, and the protection index, from Fig. 10, is 25. Both the critical O_2 tension and the protection index are measures of the degree of protection afforded to the enzyme by the carrier systems present but, since they are determined by

separate experiments, are mutually corroborative. The relevant facts for materials A, B and C are set out in Table VII.

Table VII

Material	Q_{O_2}	Q_{MB}	Cytochrome content	Critical O_2 tension	Protection index
Untreated sediment A	164	110	× × ×	15–20 %	20
Autolysed material B	153	324	×	15 %	15
Digested material C	0	474	—	0	0

Effect of the oxygen tension present during growth of the organism

In order to grow cultures on the surface of agar under any desired O_2 tension, the following technique was devised: a Roux bottle containing broth-agar is sterilized and, while the agar is still liquid, a small sterile glass boat is slipped in through the neck and embedded in the agar. When cool, the agar is inoculated as usual and 0·5 ml. 10 % NaOH put in the boat. The cotton-wool plug is replaced by a sterile rubber bung fitted with an inlet tube plugged with sterile cotton-wool. The bottle is then connected to a three-way tap connected, on one side to a pump and on the other to an aspirator containing the desired gas mixture. The Roux bottle is then filled with the gas mixture by partial evacuation followed by connexion with the aspirator. If the bottle is one-third evacuated each time and the complete operation repeated some 15 times, almost complete exchange will be effected. When full the bottle is clamped off, disconnected and then reconnected to an aspirator containing O_2. A water-valve is put in between to prevent mixture of the gases. The whole apparatus is now incubated and as O_2 is used up by the growing organisms, the evolved CO_2 will be absorbed by the NaOH in the boat and O_2 will be sucked in from the aspirator to keep the O_2 tension constant.

In this manner cultures have been grown in the presence of O_2-N_2 mixtures containing 100, 80, 50, 20 and 0 % O_2. In every case, the cultures have been

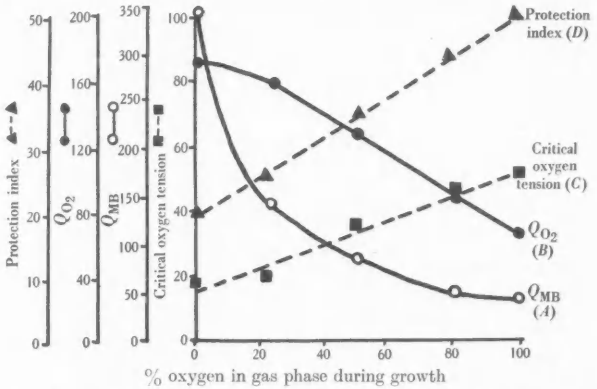

Fig. 11. Effect of oxygen tension present during growth on the properties of *Bact. coli* in regard to formate oxidation. *A*, Q_{MB}; *B*, Q_{O_2}; *C*, critical oxygen tension; *D*, protection index.

prepared in washed suspension as usual and the following quantities determined: Q_{MB}; Q_{O_2} in air during linear O_2 consumption over the first 20 min., critical O_2 tension and protection index. The results are plotted in Fig. 11 against the O_2 percentage during growth.

It is seen that increasing the O_2 tension present during growth leads to (1) a decrease in dehydrogenase activity as shown by the drop in Q_{MB} correlated with (2) a decrease in Q_{O_2} and (3) an increase in both critical O_2 tension and protection index; Wooldridge *et al.* [1936] have shown that the formic dehydrogenase of *Bact. coli* undergoes negligible variation with the age of the culture so that the results are not complicated by any such effects.

A rise in the critical O_2 tension and the protection index would be brought about, from what has been shown, by a variation in the ratio of carrier systems/enzyme present. Since the cytochrome bands for the organism are weak, it has proved impossible to state whether the cytochrome content of the organism varies significantly according to the manner of growth. However, a culture that has been grown through 20 consecutive anaerobic subcultivations appears to contain essentially the same amount of cytochrome as one grown aerobically for the same period. (I am indebted to Dr T. Mann of the Molteno Institute, Cambridge, for the examination of various cultures with respect to their cytochrome contents.) If, as seems probable, the cytochrome content of the organism is approximately constant then it would follow that any rise in the critical O_2 tolerance would be brought about by inhibition of dehydrogenase activity. From the appearance of curves *A* and *C* (Fig. 11) this would appear to be the case.

When the organism is grown anaerobically, a very high Q_{MB} is found. This is not due to the presence of formic hydrogenlyase in the anaerobic culture reducing MB through the hydrogenase system as formic hydrogenlyase is not active at the experimental *p*H 6 used [*v.* Stephenson & Stickland, 1932]. Hence the high Q_{MB} must be due to a greatly increased production of the dehydrogenase under anaerobic conditions.

Absence of coenzymes I and II in material C. Adler & Svreenivasaya [1937] showed that the formic dehydrogenase of certain plants requires the presence of coenzyme I for its action. Table VIII shows the effect of adding coenzymes I and

Table VIII. *Effect of addition of coenzymes on activity of formic dehydrogenase in material C*

Tubes contain: 1 ml. *M*/10 Na formate.
1 ml. *M*/20 phosphate buffer *p*H 6·0.
1 ml. material C = 0·45 mg. dry weight.
0·2 ml. 0·5 % methylene blue.

Additions	Reduction time min.	sec.
1 ml. water	9	30
1 ml. cozymase (=0·15 mg. coenzyme I)	9	30
1 ml. coenzyme II prep. (=0·2 mg. coenzyme II)	9	20
1 ml. boiled organism (=20 mg. dry wt.)	12	0

II and boiled organism on the Q_{MB} of material C in the presence of formate. There is no significant acceleration. Gale & Stephenson [1939] have shown that the malic dehydrogenase of *Bact. coli* requires coenzyme I but that the organism grown on agar possesses only about 27 % of the optimal amount of coenzyme required to activate the dehydrogenase present—hence, in this case, addition of cozymase to the washed suspension produces a considerable acceleration of the rate of reduction of methylene blue. No such effect is obtained with formic dehydrogenase. Further, Yudkin [1933] has shown that the activity of formic dehydrogenase of *Bact. coli* does not vary with the dilution of the organism whereas the activity of other dehydrogenases, known to require coenzymes,

decreases rapidly with dilution. Thus it would appear that the activity of formic dehydrogenase of *Bact. coli* does not depend upon the presence of a coenzyme. Table IX shows the effect of adding 1 ml. of boiled material C containing 20 mg. dry weight to (*a*) malic dehydrogenase from heart muscle and from *coli*-juice [Gale & Stephenson, 1939] and (*b*) glucosemonophosphate dehydrogenase from muscle. Also the effects of adding known amounts of coenzymes I and II and boiled *Bact. coli* (20 mg. dry weight) are shown. It is obvious that material C contains no coenzyme I or II. Hence the formic dehydrogenase of *Bact. coli* does not require either coenzyme for its action.

Table IX. *Absence of coenzymes I and II from material C*

(*a*) Test for coenzyme I

Tubes contain: 1 ml. malic dehydrogenase preparation (*a*) from heart muscle, (*b*) from *coli*-juice.
1 ml. *M*/10 Na malate.
0·2 ml. 2 *M* NaCN.
1 ml. phosphate buffer *p*H 7·2.
0·2 ml. 0·5 % methylene blue.

Additions	Reduction time	
	(*a*)	(*b*)
1 ml. water	>3 hr.	>3 hr.
1 ml. cozymase (=0·15 mg. coenzyme I)	1 min. 15 sec.	2 min. 25 sec.
1 ml. boiled material C (=20 mg. dry wt.)	>3 hr.	>3 hr.
1 ml. boiled organism (=20 mg. dry wt.)	20 min. 5 sec.	—

(*b*) Test for coenzyme II

Tubes contain: 1 ml. glucosemonophosphate dehydrogenase preparation.
1 ml. phosphate buffer *p*H 7·5.
0·3 ml. *M*/5 glucosemonophosphate.
0·2 ml. 0·5 % methylene blue.

Additions	Reduction time
1 ml. water	>2 hr.
1 ml. coenzyme II (=0·2 mg. coenzyme II)	15 min. 25 sec.
1 ml. boiled material C (=20 mg. dry wt.)	>2 hr.

Summary

1. The material obtained by grinding *Bact. coli* in the Booth-Green mill has been investigated for formic dehydrogenase activity.

2. The enzyme resides in the solid part of this material and has not been removed therefrom in an active state.

3. Digestion of this sediment with trypsin gives a material very active with methylene blue in the presence of formate but unable to utilize O_2 as H-acceptor.

4. Spectroscopic examination shows that the formic dehydrogenase of the sediment reacts normally with O_2 through cytochrome *b*. This is confirmed by the effects of cyanide, which inhibits the dehydrogenase in high concentrations.

5. Autolysis of the sediment yields a material which will oxidize formate with oxygen but which is quickly inactivated.

6. Inactivation of the enzyme occurs only when the dehydrogenase is incubated aerobically with formate.

7. The inactivation is not due to the production of oxalic acid, formaldehyde or hydrogen peroxide.

8. The inactivated enzyme can be reactivated by anaerobic incubation. This effect is accelerated by the presence of formate.

9. The inactivation is accelerated by increasing the O_2 tension. If the O_2 tension is decreased sufficiently, the inactivation is abolished.

10. The inactivation is accelerated by the action of cyanide on the carrier system (cytochrome) or checked by the addition of methylene blue.

11. The digested material will react with O_2 in the presence of methylene blue but the extent of the oxidation depends upon (*a*) the concentration of the dye and (*b*) the O_2 tension.

12. It is suggested that the enzyme reacts with O_2 along two paths: (1) directly leading to inactivation of the dehydrogenase activity, and (2) indirectly through the carrier system. The latter reaction, by diverting the course of the oxygen, protects the enzyme from the direct oxidation.

13. The digested material will give a steady O_2 uptake with a high concentration of cytochrome *c* and cytochrome oxidase in the presence of a low O_2 tension.

14. *Bact. coli* grown in air can oxidize formate linearly only if the O_2 tension is less than that of air.

15. A method for growing organisms on the surface of agar under any given O_2 tension is described.

16. Increased O_2 tension during growth leads to inhibition of formic dehydrogenase formation.

17. Formic dehydrogenase of *Bact. coli* does not require coenzymes I or II for its action.

The author wishes to thank Prof. Sir F. G. Hopkins for his continued interest in this work and Dr M. Stephenson for her encouragement and help. He is indebted to Dr M. Dixon for valuable criticism and to the Medical Research Council for a personal grant.

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CXXVI. *ISO*CITRIC DEHYDROGENASE AND GLUTAMIC ACID SYNTHESIS IN ANIMAL TISSUES

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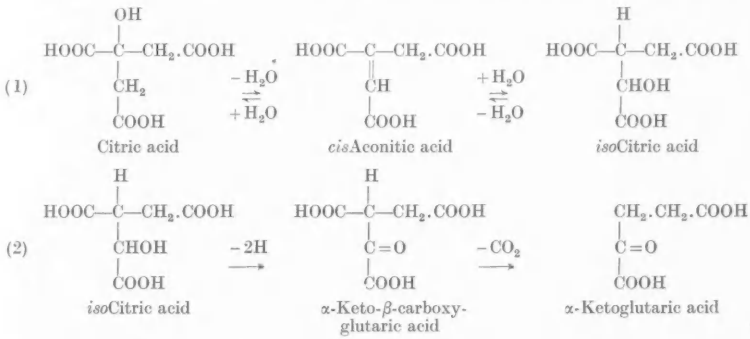
Martius & Knoop [1937; Martius, 1937; 1939] have recently shown that the biological breakdown of citric acid proceeds according to the following scheme:



Citric acid first undergoes transformation into *iso*citric acid by the action of the enzyme "aconitase". *iso*Citric acid is then dehydrogenated to the corresponding keto-acid, which is unstable and splits off CO_2 spontaneously to form α-ketoglutaric acid. Thus the old term "citric dehydrogenase" designates a mixture of enzymes and the actual dehydrogenation is brought about by an "*iso*citric dehydrogenase".

In connexion with our work on glutamic dehydrogenase and the enzymic synthesis of glutamic acid, i.e. reductive amination of α-ketoglutaric acid [Euler *et al.* 1938], we were interested in the nature of *iso*citric dehydrogenase, because it catalyses the formation of α-ketoglutaric acid and thus forms a link between carbohydrate breakdown and protein synthesis in the cells.

The history of "citric dehydrogenase", especially the work of Thunberg, Batelli & Stern, and Bernheim, is given in the monograph of Franke [1934]. More recently Andersson [1933] found that a crude cozymase preparation accelerated the reduction of methylene blue by citric acid and a plant extract, and Wagner-Jauregg & Rauen [1935, 1] described an activation of a similar system by a crude coenzyme prepared according to Warburg & Christian [1935] from red blood cells. Wagner-Jauregg & Rauen [1935, 2] also found that *iso*citric acid was a hydrogen donator for the plant "citric dehydrogenase" system.

From the point of view of our present knowledge of dehydrogenase systems it was of fundamental interest to find out what coenzyme had been active in

Andersson's and Wagner-Jauregg's experiments. It seems likely that the coenzyme preparations used by these authors contained both codehydrogenase I (cozymase, diphosphopyridinenucleotide, Co I) and codehydrogenase II (Warburg's coenzyme, triphosphopyridinenucleotide, Co II). Using pure preparations of these coenzymes we have found that Co II is the specific coenzyme for *iso*citric dehydrogenase from animal tissues, higher plants and yeast,[1] while Co I is completely inactive. The bearing of this fact on the problems connected with the importance of the *iso*citric dehydrogenase in carbohydrate breakdown and amino-acid synthesis is discussed in a later section.

On the basis of the Co II-specificity we were able to study the distribution of the *iso*citric apodehydrogenase in biological material, to purify the enzyme and to study the dehydrogenating system. The apodehydrogenase was found to be present in all animal tissues so far examined, which makes it probable that citric acid breakdown is part of a general cell reaction. It was possible to obtain the *iso*citric dehydrogenase free from "aconitase" and thus enzyme preparations were obtained which used *iso*citric but not citric acid as H donator. It was further shown that in the isolated *iso*citric dehydrogenase system CO_2 and α-ketoglutaric acid were formed as the end products, thus confirming Martius and Knoop's scheme.

In experiments with purified apodehydrogenase the system showed a typical "dilution effect", i.e. the rate of the reaction was not proportional to the enzyme concentration, but disappeared more or less completely when the enzyme was diluted to a certain degree. The analysis of this observation led us to the surprising fact that Mn^{++} was necessary for the full action of the dehydrogenase; Mg^{++} could replace the Mn^{++}, but was less active and its optimal concentration was higher than that of Mn^{++}. As yet no other dehydrogenase could be found in which Mn or Mg salts had an effect, whilst a number of other enzymic reactions, e.g. transphosphorylation [Ohlmeyer & Ochoa, 1937] and pyruvic acid decarboxylation [Euler *et al.* 1937] by yeast enzymes, are known to be activated by Mn^{++}. Concerning the mode of action of these ions in the case of *iso*citric dehydrogenase it may be possible, that they "link up" the substrate with the apodehydrogenase by salt formation. If the Mn^{++} effect involved the reaction between the codehydrogenase and the apodehydrogenase, one would expect that other dehydrogenases would be similarly activated.

According to the results described in this paper reaction (2) of the scheme given above can be written as follows:

$$(2a)\ \textit{iso}\text{Citric acid} + \text{Co II} \xrightarrow[+Mn^{++}\text{ or }Mg^{++}]{\textit{iso}\text{citric apodehydrogenase}} \alpha\text{-keto-}\beta\text{-carboxyglutaric acid} + CoH_2\ \text{II}.$$

$$(2b)\ \alpha\text{-Keto-}\beta\text{-carboxyglutaric acid} \longrightarrow \alpha\text{-ketoglutaric acid} + CO_2.$$

Reaction (2*b*) goes spontaneously and is very fast and therefore the whole reaction (2*a*+2*b*) must go to completion in the direction given by the arrows. That means that in presence of an excess of *iso*citric acid the total amount of Co II is hydrogenated, and on the other hand, in presence of an excess of Co II, or if the CoH_2 II formed is continuously reoxidized, the total amount of *iso*citric acid is converted into ketoglutaric acid. Whether reaction (2*a*) is reversible in analogy with other hydroxy-acid $\rightleftharpoons$ keto-acid reactions cannot be said, because it was not possible to study this reaction independently of reaction (2*b*).

[1] The experiments with higher plants and yeast have been done by L. Elliot and will be published separately.

Experimental

The components of the system

(*a*) *Enzyme preparations.* Acetone-dried heart muscle was used as starting material. Pig heart was freed from fat and ground in a mincer. The pulp was stirred up 3 times with twice the volume of ice-cold acetone, pressed out through muslin each time and finally dried in air by spreading out on filter paper. From the resulting stable preparation enzymes were prepared in different ways.

Enzyme A. Acetone-dried tissue was ground in a mortar with sand and 6 times its wt. of water and filtered through muslin on a Büchner funnel. The extract was dialysed for 12 hr. and centrifuged. The enzyme attacks *iso*citric acid, but not at all or only slowly citric acid.

Enzyme B. Fractionation with ammonium sulphate: 70 g. acetone-dried heart muscle were ground twice with 420 ml. 0·1 *M* Na_2HPO_4 and sand and pressed out through muslin. The *p*H of the crude extract (700 ml.) was adjusted to 6·5 by addition of 150 ml. 0·5 *M* KH_2PO_4 and 1700 ml. sat. ammonium sulphate solution were added (degree of saturation is 0·66). The protein precipitate was filtered through a thin layer of kieselguhr on a Büchner funnel and redissolved by rubbing up the filter cake with 0·1 *M* Na_2HPO_4. The resulting solution was centrifuged and the supernatant was neutralized with 50 ml. 0·5 *M* KH_2PO_4; 175 ml. sat. ammonium sulphate were then added and the precipitate formed was discarded. The filtrate was precipitated once more with an equal volume of ammonium sulphate, and the proteins remaining on kieselguhr after filtration were redissolved in 70 ml. water and centrifuged. The reddish, clear solution contains aconitase and a very active *iso*citric dehydrogenase.

Enzyme C. Fractionation with acetone: 50 g. acetone-dried heart muscle were thoroughly ground with 400 ml. water and sand. The extract (250 ml.) was precipitated at 0° with 750 ml. cold acetone; the precipitate was dissolved in 130 ml. water; the centrifuged solution (125 ml.) was precipitated at 0° with 65 ml. acetone and after centrifuging off the precipitate formed, another 65 ml. acetone were added. Both precipitates were dried with cold acetone and ether. The second fraction was used in most of the experiments and is called "enzyme C". The dry powders are nearly completely soluble in water; both fractions are rich in *iso*citric dehydrogenase but free from aconitase.

(*b*) *Coenzyme.* Codehydrogenase II was prepared by enzymic phosphorylation of cozymase according to the principle previously described [Euler & Adler, 1938]. The details of this will be published separately.

(*c*) *Substrate. iso*Citric acid was a synthetic preparation for which we wish to express our thanks to Prof. P. Karrer, Zürich. The Na salt was used in the experiments.

Citric acid as a substrate[1]

Methylene blue as acceptor. Table I shows that codehydrogenase II and flavinenzyme are necessary for methylene blue reduction when citrate is used as substrate for "enzyme B".

Exps. 2 and 3 show that the methylene blue decoloration with 40 μg. Co II was about 25 times faster than that with 250 μg. of a cozymase preparation of highest purity. It is difficult to say whether cozymase has an action in this system which is 150 times weaker than that of codehydrogenase II or if cozymase is actually inactive and the low rate obtained in Exp. 3 is due to traces of codehydrogenase II present in the cozymase preparation. At any rate the great difference in the degree of action indicates that *iso*citric dehydrogenase is practically specific for codehydrogenase II.

[1] For a preliminary report see Adler *et al.* [1938, 3].

Table I. *The "citric acid dehydrogenase" system*

0·5 *M* Na-citrate; "enzyme B"; cozymase (Co I) of highest purity, 1 mg./ml.; codehydrogenase II (Co II), 200 μg./ml.; flavinenzyme from yeast, 2·5 μg. bound lactoflavin per ml. Each Thunberg tube contained 0·25 ml. 0·5 *M* phosphate buffer, *p*H 7·6, and in a small inner tube 0·5 ml. 0·02% methylene blue, which is mixed with the other components after evacuation. Temp. 30°.

In Exp. 1 the citrate was added to the other components immediately before the experiment was started; in all other experiments citrate + enzyme + buffer + water were incubated in the open tube for 15 min. at 30°, then the other components were added and the experiment was started.

Exp. no.	Citrate ml.	Enzyme ml.	Co I ml.	Co II ml.	Flavin-enzyme ml.	Decoloration time min.
1	0·25	0·25	—	0·20	0·25	8
2	0·25	0·25	—	0·20	0·25	2
3	0·25	0·25	0·25	—	0·25	53
4	—	0·25	—	0·25	0·25	240
5	—	0·25	0·25	—	0·25	205
6	0·25	0·25	—	0·25	—	79
7	0·25	0·25*	—	0·25	0·25	240
8	0·25	0·25	—	0·25	0·25†	78
9	0·25	0·25	—	0·25‡	0·25	240

* Boiled after incubation with citrate. † Boiled flavinenzyme.
‡ Alkali-heated Co II.

A comparison between Exps. 1 and 2 shows that the decoloration time is remarkably shorter when the citrate is incubated with the enzyme for some time before the other components are added and the dehydrogenation is started. This effect is explained by the Martius-Knoop scheme: during incubation the transformation citric acid $\rightleftharpoons$ *iso*citric acid takes place and possibly the equilibrium is reached within the incubation time, whilst in the experiment without incubation there is not enough *iso*citric acid formed to reach the optimal concentration for the dehydrogenase reaction.

Oxygen as acceptor. Fig. 1 shows that the system citrate+enzyme B+ codehydrogenase II takes up O_2 when it is completed by flavinenzyme and methylene blue.

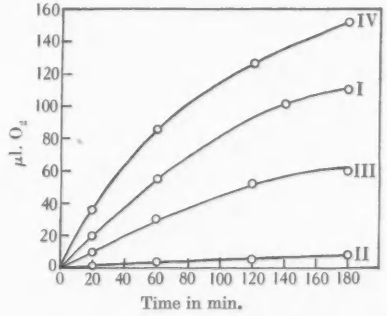


Fig. 1. Aerobic breakdown of citric acid. Curve I: 0·25 ml. *M*/2 citrate, 1 ml. "enzyme B", 0·5 ml. flavinenzyme, 0·25 ml. veronal buffer *p*H 7·66, 0·5 ml. methylene blue 1:5000; after 15 min. incubation at 30°, 0·25 ml. (=25 μg.) Co II were added from the side bulb of the manometer flask. Curve II: with heated flavinenzyme. Curve III: without methylene blue. Curve IV: with double amount of flavinenzyme. No O_2 uptake was obtained in controls without substrate, without Co II, with heated enzyme and with Co I instead of Co II. The centre cup of the vessels contained 0·2 ml. 10% KOH.

In the absence of methylene blue the O_2 uptake is slower, because then the rate is limited by the autoxidizable fraction of our flavinenzyme preparation, i.e. the flavinphosphate protein fraction, whilst the flavin-adenine-dinucleotide protein fraction [Warburg & Christian, 1938; Haas, 1938) will react rapidly only if methylene blue is added. In absence of flavinenzyme the reaction is extremely slow; this means that "enzyme B" does not contain appreciable amounts of "diaphorase II" [Adler *et al.* 1939], a flavoprotein of animal tissues, which transports hydrogen from CoH_2 II to acceptors like methylene blue. This conclusion was confirmed by direct spectrophotometric determination in the system CoH_2 II + "enzyme B" + O_2; with the same technique it was shown that "enzyme B" was relatively rich in "diaphorase I", the CoH_2 I-specific hydrogen transporting enzyme.

Separation of aconitase and iso*citric dehydrogenase*

When "enzyme B" was dialysed in a cellophane tube against running water for 20 hr., the activity towards citric acid was practically abolished; the activity towards *iso*citric acid was decreased too, but could be restored to the level of the non-dialysed enzyme by addition of Mn^{++} (cf. p. 1037), whilst citrate was not attacked even in presence of Mn^{++} or of boiled undialysed enzyme; but when citrate was incubated with non-dialysed "enzyme B" for 15 min., the mixture deproteinized by heating and used as a substrate in a Thunberg experiment containing the dialysed "enzyme B", the MB was rapidly decolorized. Therefore, the aconitase must have been inactivated by dialysis.

Another way of separating aconitase is to precipitate the crude enzyme with acetone. Thus "enzyme C" which is a stable acetone powder, is completely inactive with citrate though highly active with *iso*citrate, especially after addition of Mn^{++}. Sensitivity to acetone treatment is also characteristic of fumarase [Clutterbuck, 1928], but according to Martius [1939] aconitase and fumarase are different enzymes.

The iso*citric dehydrogenase system*

(*a*) *Methylene blue as acceptor.*

The dehydrogenation of *iso*citric acid by one of the enzymes A, B, or C with methylene blue as H acceptor needs the addition of the same components as if citric acid is used as a substrate, namely codehydrogenase II and flavinenzyme. Codehydrogenase II cannot be replaced by cozymase.

Fig. 2 shows the dependence of the rate of methylene blue reduction on the codehydrogenase II concentration and in Fig. 3 the influence of increasing amounts of yeast flavinenzyme is shown. The relation between the rate of *iso*citric acid dehydrogenation and the amount of apodehydrogenase is described in a later section in connexion with the experiments on Mn^{++} activation. Here it may be mentioned that proportionality between rate and apodehydrogenase concentration exists only if the system contains an optimal amount of Mn^{++}.

Substrate affinity. Experiments on the influence of *iso*citric acid concentration showed that the rate was still optimal when the concentration of the substrate was as low as 5×10^{-5} *M*, assuming that the *iso*citric acid preparation used contained 50% of the natural form. Thus, in a Thunberg experiment with 2·0 ml. total volume, 19 μg. *iso*citric acid (equivalent to 37 μg. methylene blue) were sufficient to decolorize the methylene blue (20 μg.) at the optimal rate. Since a further decrease in the amount of methylene blue would have introduced considerable error in the measurement of the decoloration time, experiments

with still lower amounts of *iso*citric acid could not be done. In spectrophotometric experiments (cf. p. 1036), the *iso*citric acid concentration could be lowered to $1{\cdot}25 \times 10^{-5}$ *M*, without a distinct decrease in the rate of dehydrogenation. Thus, the Michaelis constant K_m must have a value $< 1{\cdot}25 \times 10^{-5}$ *M*, which means that the affinity of *iso*citric acid for the apodehydrogenase is extremely high.

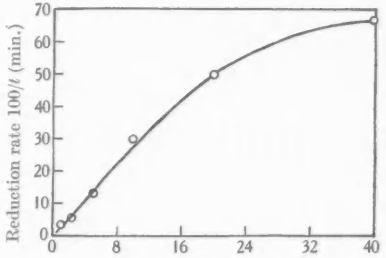


Fig. 2.

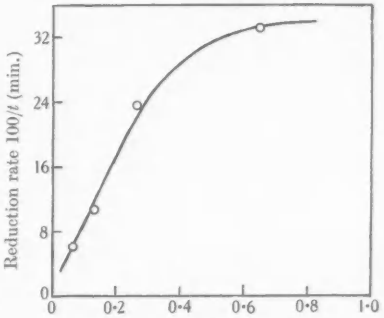


Fig. 3.

Fig. 2. *iso*Citric acid dehydrogenation; effect of coenzyme concentration. Thunberg technique. 0·1 ml. "enzyme B" was used in each experiment.

Fig. 3. *iso*Citric acid dehydrogenation; effect of flavinenzyme concentration. Thunberg technique. 0·1 ml. "enzyme B" was used in each exp.

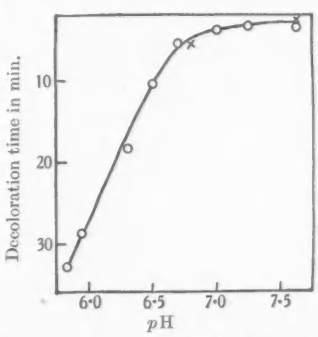


Fig. 4. *iso*Citric acid dehydrogenation; effect of *p*H. Thunberg technique. 0·1 ml. "enzyme C", 20 μg. Co II, 0·25 ml. flavinenzyme and 2 mg. *iso*citric acid were used in each exp. ○ = veronal buffer; × = glycine buffer.

Influence of p*H*. Fig. 4 shows the influence of the *p*H of the solution on the rate of *iso*citric acid dehydrogenation, determined by the Thunberg technique. It is important to say that phosphate buffer could not be used in these experiments as it was found that phosphate ions inhibit the dehydrogenase reaction (cf. p. 1040) and that the % inhibition varies considerably with the *p*H. Therefore, the *p*H curve with phosphate buffer showed a maximum between *p*H 6 and 6·5, where the phosphate inhibition is relatively small, and a minimum between *p*H 7 and 8, where phosphate exerts a strong inhibiting action. By the use of

veronal and glycine buffer this difficulty was overcome and a normal *p*H curve, analogous to other animal dehydrogenases was found. The rate is high at *p*H 7–7·5 and falls off rapidly below *p*H 6·5; the values at alkaline reaction, where the curve also falls off, are not shown in the figure, because in this region the rate may be decreased by destruction of the codehydrogenase.

(*b*) *Oxygen as acceptor.*

The components of the system. A mixture of *iso*citric acid, apodehydrogenase and codehydrogenase II takes up O_2 if the transfer of H from the reduced coenzyme to the O_2 is made possible by the addition of flavinenzyme. A further addition of methylene blue increases the rate of O_2 uptake very much, because in presence of this dye even the "new", i.e. not readily autoxidizable, flavoprotein, present in the flavinenzyme preparation, is utilized (Table II).

Table II. *Aerobic dehydrogenation of* isocitric *acid*

The total system contained 0·25 ml. apodehydrogenase solution (corresponding to 4 mg. acetone powder "enzyme C"); 0·20 ml. (=20 μg.) codehydrogenase II; 0·25 ml. flavinenzyme from yeast (corresponding to 0·8 μg. bound lactoflavin); 0·50 ml. methylene blue 1:1000; 0·25 ml. veronal-acetate buffer (Michaelis), *p*H 7·66, and 0·25 ml. Na *iso*citrate (corresponding to 5 mg. *iso*citric acid); the total volume was 2·25 ml. The centre cup of the Warburg vessels contained 0·3 ml. 7% KOH, absorbed on filter paper. The substrate was added from the side bulb.

	μl. O_2 in 20 min.
Total system	49
No substrate	0
With heated enzyme	0
No coenzyme	0
No flavinenzyme	3
No methylene blue	8
With Co I instead of Co II	0

The oxygen equivalence. The amount of O_2 taken up by a certain amount of our *iso*citric acid preparation agrees rather well with the theory: one pair of H atoms is taken away from the substrate, dihydrocoenzyme is formed and the 2H transported to the O_2, giving H_2O_2; the latter is split by catalase, the presence of which in the apodehydrogenase preparation was shown in separate experiments, and 1/2 mol. O_2 is liberated again. Thus an actual uptake of 1/2 mol. O_2 per mol. *iso*citric acid would be expected.

We found in several experiments with "enzyme C" an O_2 uptake a little higher than calculated, e.g. for 1·9 mg. *iso*citric acid an O_2 uptake of 65 μl. (calculated 56 μl.) and for 3·8 mg. *iso*citric acid an oxygen uptake of 125 μl. (calculated 112 μl.) was found. The small discrepancy between the theoretical and the experimental values can have several reasons; it can be at least partially explained by the fact that our *iso*citric acid preparation contained an unknown amount of the corresponding lactone which has a lower mol. wt. and so will give higher values for the O_2 uptake than the same amount of free acid. With crude enzyme preparations, e.g. "enzyme A", the O_2 uptake was usually lower than calculated, which possibly indicates that part of the reduced coenzyme is used up in an anaerobic reaction; one could assume that *cis*aconitic acid which, in presence of crude enzymes, is in equilibrium with *iso*citric acid, might act as H acceptor, forming tricarballylic acid. However, no direct proof of the existence of such a reaction has been found as yet.

CO_2 as a reaction product. If in the aerobic experiment no KOH is used in the centre cup, the negative pressure is replaced by a positive one, showing that CO_2

is formed during the reaction. The respiratory quotient was determined according to Warburg & Yabusoe [1924]. In experiments in which the reaction mixture was the same as that given in Table I, but with 4 mg. substrate, after 100 min., when the reaction was finished, 140 μl. O_2 had been taken up and 278 μl. CO_2 had been formed. Thus, the quotient mol. CO_2/mol. $O_2 = 2$, i.e. for each mol. *iso*citric acid 1/2 mol. O_2 is taken up and 1 mol. CO_2 is formed. This result is in complete agreement with Martius and Knoop's scheme.

α-Ketoglutaric acid as a reaction product. Definite proof of the mechanism of the isolated dehydrogenase reaction as given by scheme (2) consists in the isolation of α-ketoglutaric acid.

The collected reaction mixtures of five aerobic experiments, corresponding to those given in Table II, were deproteinized with 2% trichloroacetic acid and centrifuged. The supernatant solution was freed from methylene blue by slow filtration through a layer of kieselguhr and concentrated *in vacuo* to 5 ml. After addition of 3 ml. sat. 2:4-dinitrophenylhydrazine in 2*N* HCl, crystals of a 2:4-dinitrophenylhydrazone settled out. They were collected after standing overnight and washed with 2*N* HCl. For purification the hydrazone was taken up in diluted Na_2CO_3, in which it was completely soluble, and reprecipitated with HCl. The substance was identified as the 2:4-dinitrophenylhydrazone of α-ketoglutaric acid by M.P. (218°) and mixed M.P. (218°); the 2:4-dinitrophenylhydrazone of the pure acid melted at 219°. The yield was 12 mg. of the crude substance.

Summarizing the preceding qualitative and quantitative results, it has been shown that the aerobic dehydrogenation of *iso*citric acid by the isolated system proceeds in the following way:

(2*a*) *iso*Citric acid + Co II $\xrightarrow[\text{dehydrogenase}]{\text{apo-}}$ α-keto-β-carboxyglutaric acid + COH_2 II.

(2*b*) α-Keto-β-carboxyglutaric acid → α-ketoglutaric acid + CO_2.

(3) CoH_2 II + O_2 $\xrightarrow{\text{flavinenzyme + MB}}$ Co II + H_2O_2.

(4) H_2O_2 $\xrightarrow{\text{catalase}}$ $H_2O + \frac{1}{2}O_2$.

The sum of these reactions will be

(5) *iso*Citric acid + 1/2 O_2 → α-ketoglutaric acid + CO_2 + H_2O.

The catalytic components of the system which brings about the primary anaerobic step of the dehydrogenation, i.e. *iso*citric apodehydrogenase and codehydrogenase II, are present probably in all animal cells. But for the transport of H from CoH_2 II to O_2 we used flavinenzyme from yeast, because our apodehydrogenase preparation did not contain a carrier enzyme capable of reacting with CoH_2 II. However, as Adler *et al.* [1939] have shown, animal tissues do in fact contain an enzyme, "diaphorase II", which transfers H from CoH_2 II to acceptors like methylene blue and probably also to cytochrome; hence the possibility of aerobic dehydrogenation of *iso*citric acid in animal tissues is evident. Actually, citric acid has been shown to increase the O_2 uptake of various animal tissues [Batelli & Stern, 1911; Krebs & Eggleston, 1938].

Besides the aerobic way there is another possibility for continuous dehydrogenation of *iso*citric acid in the cells, namely the anaerobic reoxidation of the CoH_2 II by iminoglutaric acid, i.e. by α-ketoglutaric acid + NH_3, catalysed by glutamic apodehydrogenase. This reaction will be discussed in a later section.

(*c*) *Spectrophotometric experiments.*

The primary reaction (2*a*) between *iso*citric acid and codehydrogenase II in presence of the apodehydrogenase can be easily studied by spectrophotometric determination of the characteristic absorption band with maximum at 340 mμ of the dihydro-codehydrogenase II.

In Fig. 5, Curve I shows the hydrogenation of a certain amount of codehydrogenase II by an excess of *iso*citric acid, catalysed by "enzyme C". In the parallel experiment (Curve II), the same amount of Co II was used, but the hydrogenation was brought about by hexosemonophosphate in presence of hexosemonophosphate apodehydrogenase from yeast. This reaction is known to be irreversible, i.e. if an excess of substrate is used, the total amount of coenzyme is hydrogenated. The end extinctions in Exps. I and II are equal; thus the hydrogenation of the Co II was complete in the *iso*citric system.

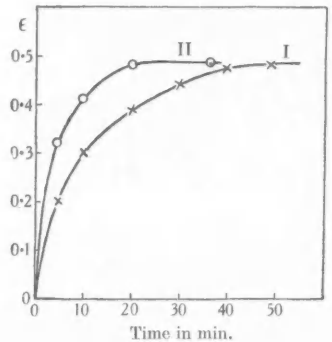


Fig. 5.

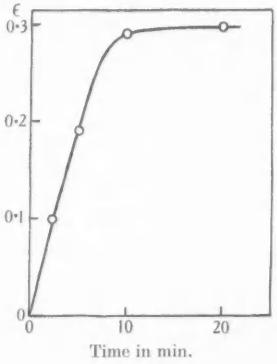


Fig. 6.

Fig. 5. Hydrogenation of codehydrogenase II by excess of *iso*citric acid (Curve I) and hexosemonophosphate (Curve II). Curve I: 0·19 mg. ($=10^{-3}$ mM) *iso*citric acid, 0·1 ml. "enzyme C", 0·5 ml. ($=0{\cdot}17\times10^{-3}$ mM) Co II, 0·5 ml. veronal buffer. Curve II: 0·2 ml. $M/10$ hexosemonophosphate, 0·1 ml. hexosemonophosphate dehydrogenase, 0·5 ml. Co II, 0·3 ml. $M/2$ phosphate buffer. Total volume 4 ml. The extinction (ϵ) at $\lambda=334$ mμ, indicating the formation of CoH_2 II, was measured photoelectrically.

Fig. 6. Hydrogenation of Co II by less than the equivalent amount of *iso*citric acid. $0{\cdot}2\times10^{-3}$ mM *dl-iso*citric acid and $0{\cdot}13\times10^{-3}$ mM. Co II were used. CoH_2 II found: $0{\cdot}11\times10^{-3}$ mM (calc. $0{\cdot}10\times10^{-3}$).

When an excess of codehydrogenase was used, the dihydro-compound formed was equivalent to the *iso*citric acid (Fig. 6).

These experiments show that the reaction between *iso*citric acid and Co II goes to completion. This can be explained by the following alternative assumptions: (1) the primary reaction (2*a*) is irreversible: (2) reaction (2*a*) is reversible and gives an equilibrium, which is rapidly disturbed by the decomposition of the keto-acid formed (2*a*+2*b*). The observation that in aerobic experiments CO_2 output begins immediately shows that the α-keto-β-carboxyglutaric acid actually is rapidly decarboxylated. Thus, the conditions for a reaction according to the second assumption seem to be given. Then, concerning the rate of the spontaneous decarboxylation, it could be said that it must be rather high, because in spectrophotometric as well as in methylene blue experiments the rate

of dehydrogenation seemed to be dependent solely on the enzyme concentration, even when this was relatively high. If the decarboxylation were a slow reaction, and if assumption (2) were correct, it should have limited the rate of the dehydrogenation. A more detailed study of the kinetics of this reaction would be of interest.

Fig. 7 represents a spectrophotometric proof of the Co II-specificity of *iso*citric apodehydrogenase. Pure cozymase (Co I) gave no reduction band, but after addition of Co II the extinction was raised to a value corresponding to complete transformation into CoH_2 II. When pyruvate was added at the end of the reaction, no change occurred. This rules out the possibility that the reaction might involve a dephosphorylation of CoH_2 II to CoH_2 I, because the latter compound would have been reoxidized by the pyruvate and lactic apodehydrogenase which was present in the enzyme preparation.

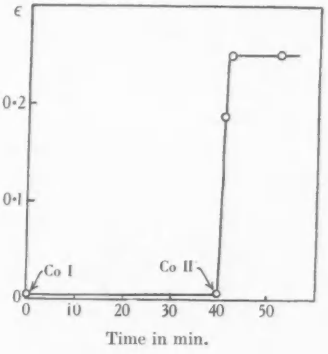


Fig. 7.

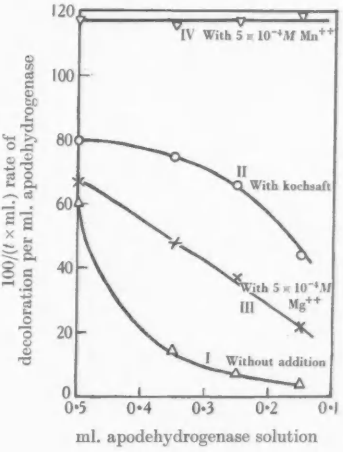


Fig. 8.

Fig. 7. Codehydrogenase II-specificity of *iso*citric dehydrogenase.

Fig. 8. Effect of enzyme dilution; activation by kochsaft, Mg^{++} and Mn^{++}. The curves refer to the Thunberg exp. of Table III.

Mn^{++} and Mg^{++} as complements of the isocitric dehydrogenase

Methylene blue experiments. As mentioned before, it was observed that the *iso*citric dehydrogenase system showed a typical "dilution effect", especially if purified enzymes were used. This may be illustrated by the experiments given in Table III and by Curve I in Fig. 8. The table contains the original figures for the decoloration times obtained with different amounts of apodehydrogenase, whilst in the curves of Fig. 8 the reciprocal values of the decoloration times, calculated for 1 ml. apodehydrogenase solution, are plotted against the amounts of apodehydrogenase solution used. It is seen that Curve I falls off sharply with decreasing amounts of apodehydrogenase; if there had been no dilution effect the curve would have remained horizontal.

From this observation one must conclude that the apodehydrogenase solution contained besides the enzyme another substance, essential for the reaction and

present in a suboptimal concentration. If this substance were thermostable, then kochsaft of the apodehydrogenase solution, added to the system, should be able to remove the dilution effect. In fact, kochsaft caused a great activation, especially at low enzyme concentrations, i.e. it partially removed the dilution effect. Further, it was shown that the ash of the apodehydrogenase preparation still activated the reaction; the activating substance must therefore have been inorganic. Mn salt was then found to activate enormously and to bring about a complete proportionality between rate and apodehydrogenase concentration. This is demonstrated in Table III and by Curve IV in Fig. 8. Mg^{++} had a similar action but the activation was less and a dilution effect was still found.

Table III. *Effect of Mn^{++} and Mg^{++} on the* isocitric *dehydrogenase*

The apodehydrogenase solution used contained 1·8 mg. "enzyme C" per ml. The reaction mixture contained 0·1 ml. (=0·19 mg.) *iso*citric acid as Na salt, apodehydrogenase solution in the varying amounts given in the first column, 0·20 ml. (=0·20 μg.) Co II, 0·25 ml. flavinenzyme, 0·25 ml. veronal buffer, *p*H 7·66, 0·5 ml. methylene blue 1 : 5000, plus various additions as indicated. Total volume 2·0 ml.

Apodehydrogenase ml.	Decoloration time: No addition min. sec.	Kochsaft from 3·6 mg. "enzyme C" min. sec.	Ash from 1·5 mg. "enzyme C" min.	$MgSO_4$ 5×10^{-4} *M* min. sec.	$MnSO_4$ 5×10^{-4} *M* min. sec.
0·50	3 20	2 30	—	3 0	1 45
0·35	20 0	3 50	—	6 0	2 30
0·25	52 0	6 0	11	10 50	3 20
0·15	140 0	16 0	—	30 30	5 30

Controls with Mg^{++} and Mn^{++} and without substrate or without Co II or without flavinenzyme were negative.

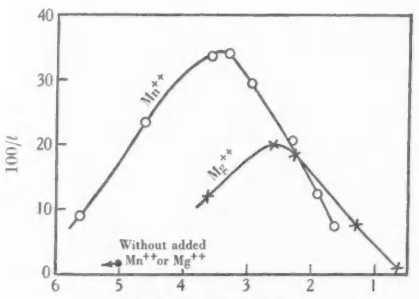


Fig. 9.

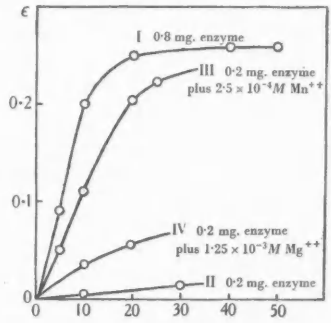


Fig. 10.

Fig. 9. Effect of Mg^{++} and Mn^{++} concentration. Thunberg experiments with 0·25 ml. "enzyme C" (=0·45 mg. dry powder) and the other components as given in Table III.

Fig. 10. Effect of enzyme dilution and of Mn^{++} and Mg^{++} on the rate of CoH_2 II-formation. 0·2 mg. *iso*citric acid and 70 μg. Co II were used in all experiments. Amounts of "enzyme C" and additions of $MnSO_4$ and $MgSO_4$ as indicated.

Fig. 9 shows that the activating action of Mn and Mg salts is dependent on their concentration. For both ions distinct concentration optima exist, which

were found to be about 5×10^{-4} *M* for Mn^{++} and about $2{\cdot}5 \times 10^{-3}$ *M* for Mg^{++}. The maximal activity brought about by Mg^{++} is only 59% of that caused by Mn^{++}.

If suboptimal amounts of Mn^{++} and Mg^{++} are added simultaneously, the activity was only very slightly higher than the activity found with Mn^{++} alone. This seems to indicate that Mn^{++} and Mg^{++} are involved in the same reaction and their action is in principle the same, but Mn^{++} may have a greater affinity for the enzyme. $CaCl_2$, $ZnSO_4$ and $CdSO_4$ had no effect.

As is known, Mg and Mn ions are normal constituents of tissues, and therefore it is likely that these ions actually represent the natural complements of the *iso*citric dehydrogenase system.

Spectrophotometric experiments. The spectrophotometric determination of the rate of CoH_2 II-formation shows that the Mn^{++} and Mg^{++} effect is involved in the primary step of the dehydrogenation and has nothing to do with the transport of H from CoH_2 II to the acceptor. Curve I in Fig. 10 gives the rate of CoH_2 II-formation with 0·8 mg. of the acetone powder "enzyme C". When 1/4 of this amount was used (Curve II), the rate was much less than 1/4 of the rate given in Curve I: within 10 min. only about 1/15 as much CoH_2 II was formed. Thus, the "dilution effect", shown with the Thunberg technique, is confirmed by the spectrophotometric experiment. Curve III shows the effect of Mn^{++} and Curve IV that of Mg^{++} on the rate of CoH_2 II-formation with the lower apodehydrogenase concentration.

Concerning the mechanism of Mn^{++} and Mg^{++} action it has been already mentioned that the most probable assumption may be that the ions favour the combination of apodehydrogenase and substrate. We have tested a number of other dehydrogenase systems, including lactic, malic, glutamic and hexosemonophosphate dehydrogenases, without finding any Mn or Mg effect. Therefore, the effect probably does not involve the equilibrium apodehydrogenase + codehydrogenase $\rightleftharpoons$ holodehydrogenase, which occurs in all these systems. Another possibility to be taken into consideration is an acceleration of the decarboxylation of the primary reaction product, α-keto-β-carboxyglutaric acid, by Mn^{++} and Mg^{++}. According to the assumption discussed on p. 1037, the primary dehydrogenation reaction (2*a*) is reversible and proceeds to completion only if the keto-acid decomposes. Therefore, dependence of the decarboxylation on Mn^{++} would result in the formation of only a small amount of CoH_2 II in systems deprived of Mn^{++}. There is, however, experimental evidence against the view that Mn^{++} affects the decarboxylation. If that were the case, the dehydrogenation should proceed, even in the absence of Mn^{++}, if a ketone fixative is added to combine with the keto-acid and so disturb the assumed equilibrium (2*a*). With the spectrophotometric technique it was, however, shown that dimedon does not accelerate the CoH_2 II-formation in a Mn^{++}-poor system, but that addition of Mn^{++} then had the known effect.

Inhibitors

Iodoacetic acid. The rate of methylene blue decoloration in the complete *iso*citric dehydrogenase system was inhibited 97% by *M*/100 and 75% by *M*/1000 iodoacetic acid. This effect was confirmed with the spectrophotometric technique, although here higher concentrations of iodoacetic acid seemed to be necessary to bring about a similar inhibition.

Thus *iso*citric dehydrogenase belongs to the group of apodehydrogenases which are inhibited by iodoacetic acid: triosephosphate and alcohol apodehydro-

genase from yeast [Adler *et al.* 1938, 2], triose dehydrogenase of animal tissues [Green *et al.* 1937], succinic dehydrogenase [Hopkins *et al.* 1938] and, to a less extent, alcohol dehydrogenase from liver [Adler *et al.* 1938, 2] and lactic dehydrogenase from yeast (unpublished experiment). All the other dehydrogenases were found to be resistant to iodoacetic acid. According to our present knowledge, sensitivity to this substance indicates the presence in the enzyme molecule of SH-groups which are essential for activity.

Pyrophosphate. The *iso*citric acid dehydrogenation is strongly inhibited by pyrophosphate; addition of Mn^{++} can, however, abolish this inhibition (Table IV).

Table IV. *Pyrophosphate inhibition*

Thunberg experiments with 0·2 mg. *iso*citric acid, 4 mg. "enzyme C", 20 μg. Co II, flavin-enzyme, veronal buffer. Total volume 2·0 ml.

Addition	Decoloration time min.	sec.
—	2	30
0·2 ml. $M/10$ $Na_4P_2O_7$ (neutralized)	20	0
5×10^{-4} M Mn^{++}	2	0
$Na_4P_2O_7$ plus Mn^{++}	2	0

From these experiments it becomes clear that the pyrophosphate inhibition is due to combination with the Mn^{++} or Mg^{++} present in the enzyme preparation.

As shown by Leloir & Dixon [1937], pyrophosphate also inhibits succinic dehydrogenase and it is suggested by these authors that pyrophosphate may compete with the substrate for the affinity centres of the enzyme, just as is assumed for malonate. Our experience with *iso*citric dehydrogenase indicated that the pyrophosphate inhibition of succinic dehydrogenase might be due to Mn or Mg removal. However, in connexion with other experiments, it was found in this institute [Euler & Hellström, 1939], that Mn did not influence the succinic dehydrogenase. Thus, the mechanism of pyrophosphate inhibition actually seems to be different in the two systems.

Phosphate. As mentioned before (cf. p. 1033), phosphate exerts a distinct inhibition, which is higher in the alkaline than in the acid region (Table V).

Table V. *Phosphate inhibition*

0·1 ml. "enzyme B" and different amounts of phosphate buffer were used; the other components were the same as given in Table IV. The *p*H was determined after the experiment.

*p*H	Phosphate concentration (M)	Rate of MB-decoloration ($100/t$)	Inhibition %
7·56	$1·25 \times 10^{-2}$	16	—
7·54	$1·88 \times 10^{-2}$	10	37·5
7·58	$2·50 \times 10^{-2}$	5	56
7·56	$6·25 \times 10^{-2}$	2·8	82
6·64	$1·25 \times 10^{-2}$	11·8	—
6·63	$2·50 \times 10^{-2}$	8·7	26
6·63	$6·25 \times 10^{-2}$	7·7	34

Phosphate inhibition was observed by Theorell [1935] in the complete hexosemonophosphate system and confirmed by Adler & Günther [1938] by the spectrophotometric method, thus showing that phosphate affects the primary reaction between substrate, apo- and co-dehydrogenase. A competition by the phosphate with either the substrate or the Co II for the apoenzyme may be the

reason for the inhibition. Similarly, in the *iso*citric system, phosphate may compete with the Co II. Furthermore, as manganese phosphate is only slightly soluble, the phosphate inhibition could be explained by precipitation of Mn^{++}.

No inhibition was found with cyanide, malonate, fluoride and oxalate.

Distribution

*iso*Citric apodehydrogenase has been found in all animal tissues so far examined. The tissues were minced and dried with acetone, the acetone preparations were extracted by grinding with 5 times the weight of water and sand and the extracts were dialysed for 5–20 hr. In the cases of brain and spleen the apodehydrogenase was not found in extracts prepared in this way, but its presence could be shown in extracts from fresh tissue. Table VI gives the relative amounts of the apodehydrogenase in various tissues as determined by Thunberg experiments. Mn salt was added to the reaction mixture.

Table VI. *Distribution of* iso*citric apodehydrogenase*

Tissue	Relative concentration
Heart	+ + +
Liver	+ + +
Kidney	+ + +
Adrenal gland	+ + +
Ovary	+ +
Intestine	+ +
Muscle	+
Brain	+
Lung	+
Testis	+
Jensen sarcoma [cf. Euler *et al.* 1939]	+
Spleen	(+)

The ratio of the activities of *iso*citric and glutamic apodehydrogenases is different for different tissues. Furthermore, the activities of two purified enzyme solutions, the one prepared from liver and the other from heart muscle, were determined spectrophotometrically in the systems (*a*) *iso*citric acid + Co II, and (*b*) CoH_2 II + ketoglutaric acid + NH_3. When the two enzymes were diluted in such a way that they had the same activity in system (*a*), the ratio of their activities in system (*b*) was 1:4, Therefore *iso*citric and glutamic apodehydrogenases are not identical.

The formation of glutamic acid from isocitric acid

Recently it was shown that the specific glutamic dehydrogenase catalyses the reductive amination of α-ketoglutaric acid [Euler *et al.* 1938]. Both Co I and Co II were found to act as coenzymes of this apodehydrogenase. Therefore, it follows that *iso*citric acid can be converted to glutamic acid if *iso*citric apodehydrogenase, Co II, glutamic apodehydrogenase and NH_3 are present. The following reactions occur:

(2*a*) *iso*Citric acid + Co II $\xrightarrow{\text{isocitric apodehydrogenase}}$ α-keto-β-carboxyglutaric acid + CoH_2 II.

(2*b*) α-Keto-β-carboxyglutaric acid → α-ketoglutaric acid + CO_2.

(6) α-Ketoglutaric acid + NH_3 → α-iminoglutaric acid + H_2O.

(7) α-Iminoglutaric acid + CoH_2 II $\xrightarrow{\text{glutamic apodehydrogenase}}$ glutamic acid + Co II.

The sum of these reactions is

(8) *iso*Citric acid + NH_3 → glutamic acid + H_2O + CO_2.

Thus, in the *iso*citric system, not only the substrate for the glutamic acid synthesis but also the H in the form of CoH_2 II is available for the reductive amination. The reaction represents an oxidoreduction between *iso*citric acid and α-iminoglutaric acid, catalysed by Co II, which oscillates between the two specific apodehydrogenases. This is a new example of the validity of the "two enzyme scheme" for coenzyme-dependent oxidoreductions, which was proposed previously for the oxidoreductions between triosephosphate and acetaldehyde or pyruvic acid and similar reactions [cf. Euler *et al.* 1936; Adler *et al.* 1938, 1].

The experimental evidence for the mechanism given above is based on the observation that not only *iso*citric but also glutamic dehydrogenase uses Co II as coenzyme. It has been shown [Euler *et al.* 1938] that glutamic apodehydrogenase from liver catalyses the dehydrogenation of both CoH_2 I and CoH_2 II by iminoglutaric acid. Since then we have been able to confirm this fact with glutamic apodehydrogenase from other animal tissues. Thus, it becomes clear that the non-specificity of glutamic apodehydrogenase with respect to the codehydrogenases is common for animal tissues and that reaction (8) can be a general cell reaction for the animal body. These facts are demonstrated by the following spectrophotometric experiments.

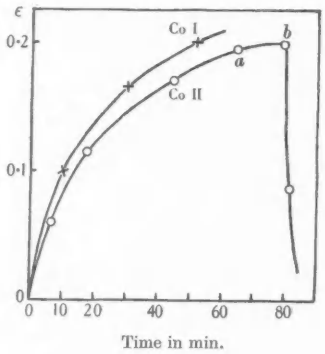


Fig. 11.

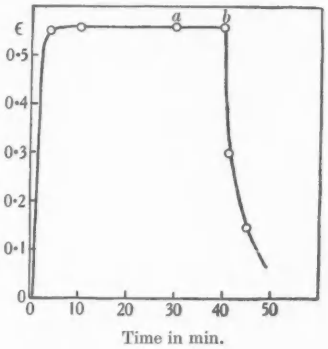


Fig. 12.

Fig. 11. Codehydrogenases I and II as coenzymes of glutamic dehydrogenase. 0·5 ml. *M*/2 glutamate, 0·1 ml. dialysed aqueous extract from acetone-liver, 0·2 ml. Co I or Co II. In the experiment with Co II were added: at (*a*) 0·1 ml. *M*/2 acetaldehyde, at (*b*) 0·1 ml. *M*/10 ketoglutarate plus 0·1 ml. *M*/2 NH_4Cl.

Fig. 12. Hydrogenation of Co II by *iso*citric acid and dehydrogenation of the CoH_2 II by α-iminoglutaric acid. 0·1 ml. *M*/10 *iso*citrate, 0·1 ml. dialysed aqueous extract of acetone kidney, 145 μg. Co II. At (*a*) 0·1 ml. *M*/10 ketoglutarate, at (*b*) 0·1 ml. *M*/2 NH_4Cl were added.

Dewan [1938], in his paper on glutamic dehydrogenase, says that this enzyme is Co I-specific. The exps. of Fig. 11, however, make it clear, in addition to the previous results [Euler *et al.* 1938], that this is not the case. The curves show the hydrogenation of Co I by glutamic acid and apodehydrogenase from liver, and

the hydrogenation of Co II by the same system. When the equilibrium was nearly reached in the experiment with Co II, acetaldehyde was added, but had no effect; if, during the reaction, a conversion of Co II into CoH_2 I had occurred, the extinction would have disappeared because the enzyme contained alcohol apodehydrogenase. However, after addition of ketoglutaric acid and NH_3 the equilibrium was pushed back and the CoH_2 II formed was reoxidized.

Fig. 12 demonstrates the oxidoreduction between *iso*citric acid and iminoglutaric acid. In this experiment Co II was hydrogenated by *iso*citrate in presence of a dialysed aqueous extract from acetone-dried kidney, which contains highly active *iso*citric as well as glutamic dehydrogenase. Addition of α-ketoglutaric acid alone had no effect, but when NH_3 was added, the extinction fell off instantaneously, showing that the CoH_2 II formed in the *iso*citric system was reoxidized by the iminoglutaric acid.

If no extra ketoglutaric acid had been added in this experiment the oxidoreduction would have occurred as well, because then the ketoglutaric acid formed from *iso*citric acid would have been aminated. But the concentration of iminoglutaric acid would have been so small that the rate of CoH_2 II-disappearance would probably have been less than the rate of re-hydrogenation of the Co II by the excess of *iso*citric acid, and no decrease of extinction would have been observed until the whole of the *iso*citric acid had been used up. It is, however, possible to demonstrate the direct transformation of *iso*citric acid into glutamic acid (Fig. 13) if an excess of Co II is used instead of an excess of *iso*citric acid. Then, in the first phase of the experiment, the total amount of *iso*citric acid is

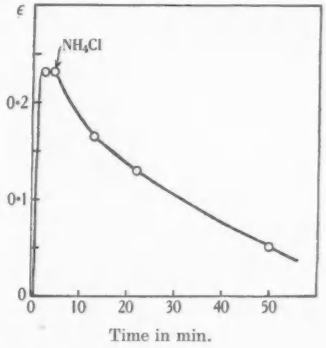


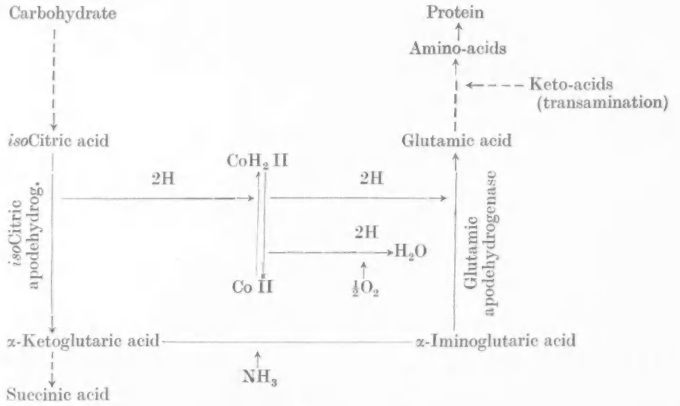
Fig. 13. Oxidoreduction between *iso*citric acid and iminoglutaric acid. $1{\cdot}8 \times 10^{-4}$ m*M* *dl-iso*citric acid, $0{\cdot}95 \times 10^{-4}$ m*M* Co II, 0·1 ml. "enzyme C", which contains glutamic besides *iso*citric apodehydrogenase.

converted into ketoglutaric acid and an equivalent amount of CoH_2 II is formed. If now NH_4Cl is added, iminoglutaric acid is formed, which dehydrogenates the CoH_2 II. "Enzyme C", which contains both apodehydrogenases, was used in this experiment.

Discussion

The mechanism of the transformation of *iso*citric acid and the function of the *iso*citric dehydrogenase system as a link between carbohydrate breakdown and

protein synthesis, can, according to the results presented in this paper, be symbolized as follows:



The products of *iso*citric acid dehydrogenation, ketoglutaric acid and CoH_2 II, can react further in two directions. Either they can be used in the glutamic dehydrogenase system for the fixation of NH_3 (glutamic acid synthesis), or H can be transferred from the CoH_2 II over diaphorase II and the cytochrome system to O_2, and the ketoglutaric acid can be broken down to succinic acid. The H necessary for the reductive amination of ketoglutaric acid can come, via CoH_2 I or CoH_2 II, from other dehydrogenase systems too, but it appears likely that the direct coupling of the *iso*citric and glutamic acid systems will be the most effective way. Ketoglutaric acid is regenerated from glutamic acid by transamination [Braunstein & Kritzmann, 1937].

According to the "citric acid cycle" theory of Krebs [cf. Krebs & Eggleston, 1938 and preceding papers] the system *iso*citric acid—ketoglutaric acid is part of a catalytic system in the oxidative breakdown of carbohydrate. If this theory is correct, codehydrogenase II is an indispensable part of the complex of cell respiration, especially the pyruvic acid oxidation, whilst codehydrogenase I is known to be necessary for the removal of the first pair of H atoms from the carbohydrate, i.e. from the triosephosphate molecule. Mn^{++}, as a complement of *iso*citric dehydrogenase, would also be involved in that part of cell respiration which concerns the breakdown of pyruvic acid. It seems possible that one could find support for the citric acid cycle theory by studying the action of Co II and Co I separately as well as of Mn^{++} on cell respiration.

Summary

1. *iso*Citric apodehydrogenase was prepared from heart muscle. This enzyme catalyses specifically the dehydrogenation of *iso*citric acid by codehydrogenase II. The substrate affinity of the enzyme is extremely high. Citric acid is used only if aconitase is present in the enzyme preparation.

2. When the system was completed with flavinenzyme, 1/2 mol. O_2 was taken up and 1 mol. CO_2 was formed per mol. *iso*citric acid, and α-ketoglutaric acid was isolated as the reaction product.

3. The system *iso*citric acid + Co II + apodehydrogenase does not react unless Mn^{++} or Mg^{++} are present; Mn^{++} is more active than Mg^{++}.

4. Iodoacetic acid and pyrophosphate are strong inhibitors, the first reacting with the apodehydrogenase, the second binding Mn and Mg ions.

5. *iso*Citric apodehydrogenase was found in all animal tissues so far examined; the highest concentrations are present in heart, liver, kidney and adrenal gland.

6. The mechanism of the conversion of *iso*citric acid into glutamic acid was demonstrated and the biological importance of this reaction discussed.

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CXXVII. ACONITASE[1]

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Since citric acid plays an important role in intermediary metabolism [Martius & Knoop, 1936; 1937; Krebs & Johnson, 1937] it was thought desirable to study in detail the reactions which citric acid may undergo in animal tissues. From the work of Martius & Knoop [1937] it is known that citric acid in the presence of tissue extracts forms an equilibrium with *cis*aconitic acid and *l-iso*citric acid:

(1) citric acid $\rightleftharpoons$ *cis*aconitic acid $\rightleftharpoons$ *l-iso*citric acid

The enzyme system bringing about the reversible hydration of *cis*aconitic acid has been shown to be different from fumarase and termed "aconitase" [Breusch, 1937]. This term will be used in this paper for the total enzyme system responsible for the reactions (1); it is left open whether one enzyme only brings about the two modes of reversible hydration of aconitic acid leading to citric acid or to *l-iso*-citric acid. The paper deals with some quantitative aspects of the reactions (1) especially with the investigation of the equilibrium between the three acids. After most of the work had been finished a paper of Martius appeared which partly covered the same ground [Martius, 1938]. The work reported in the present paper goes however beyond that of Martius in that it includes determinations of aconitic acid which Martius, lacking a suitable method, was unable to estimate.

Experimental

Preparation of cisaconitic acid

Anhydro-aconitic acid was prepared from ordinary (*trans*) aconitic acid by the method of Anschütz & Bertram [1904]. Aconitic acid was boiled with acetyl chloride until a clear solution was obtained. The deep yellow solution was evaporated to dryness *in vacuo* over NaOH, and the brown solid was crystallized several times from hot benzene. Eventually a white solid was obtained, which melted sharply at 76°.

The benzene was removed by standing *in vacuo* over paraffin oil, and the solid stored in a desiccator over H_2SO_4.

*Cis*aconitate solution was freshly prepared before use; a known amount of anhydro-aconitic acid was dissolved in ice-cold water and neutralized with $NaHCO_3$.

Quantitative determination of citric acid

The method of Pucher *et al.* [1936] was used. Citric acid is oxidized in the presence of bromine to pentabromoacetone which is subsequently converted into a coloured material by means of Na_2S, and is estimated colorimetrically. The use of dioxan as the colour stabilizer in place of pyridine was found to be much less objectionable, and did not affect the accuracy of the method. If the amount of citric acid to be determined exceeded 1 mg. the depth of colour was not strictly

[1] Preliminary note in *Chem. & Ind.* **58**, 56 (1939).

proportional. The solutions were therefore suitably diluted or else corrections were obtained from a calibration curve.

*Cis*aconitic acid did not give a colour when treated by this method.

Quantitative determination of aconitic acid

The method is based on the determination of the hydrogen required for the quantitative catalytic hydrogenation of the double bond. The catalyst used was a deposit of palladium on barium sulphate recommended by Köppen [1932], obtained from the Membranfilter G.m.b.h., Göttingen. The catalyst was suspended in water (10 mg./ml.) and a stream of H_2 bubbled through. For the manometric determination the flasks were filled as follows:

	Main compartment	Side bulb
(1)	2 ml. water 1 ml. 5% H_2SO_4 0·5 ml. Pd suspension	—
(2)	Do.	1 ml. test solution (acidified)

The manometers were filled with cylinder H_2 washed with alkaline pyrogallol and acid dichromate. Rapid equilibration occurred at 40°, and in pure solution of aconitic acid the reaction was completed in 40 to 60 min. (see Table I).

Table I. *H_2 uptake with pure aconitic acid solution in presence of palladium catalyst. Conditions as above*

Aconitic acid added = 172μl.

Time from mixing (min.)	15	30	50	70	290
Extra hydrogen uptake over "blank" (μl.)	106	153·5	170	171	170

In applying this method to the determination of aconitic acid in biological media difficulty was encountered owing to the interference of proteins with the catalyst. The choice of protein precipitant is limited to substances which are not themselves reducible. The method finally adopted was as follows. The solution to be tested was deproteinized by the addition of 1/5th volume of 5% HPO_3 (freshly prepared). The precipitate was filtered off, and an aliquot part of the clear filtrate was strongly acidified with H_2SO_4. The aconitic acid was extracted 2 hr. with ether in a Kutscher-Steudel [1903] extractor. One ml. of water was added to the ethereal extract and the ether evaporated off. The residue was made up to a known volume with 5% H_2SO_4, and the final solution tested as above.

The partition coefficient of *cis*aconitic acid between ether and water was found to be about 15:1 in favour of the aqueous phase at 20°. Under our conditions complete recovery was obtained with 2 hr. extraction.

Recovery of aconitic acid added to extract of pigeon breast muscle (dilution 1 in 5)

Mixtures were made up with varying amounts of aconitic acid as follows:

10 ml. extract + 1 ml. 5% H_2SO_4 + 4 ml. 5% HPO_3 + aconitic acid.

The recovery of aconitic acid after treatment as above is given in Table II.

The reaction is general for a double bond and works equally well with *cis*- and *trans*-aconitic acids, crotonic and fumaric acids. The latter can be estimated by re-extracting after hydrogenation and determining the succinic acid formed by the method of Szent-Györgyi as modified by Krebs [1937].

Table II. *Recovery of aconitic acid from pigeon breast muscle extract*

No.	Aconitic acid added (μl.)	H_2 uptake (μl.)	Aconitic acid recovered	% recovery
1	—	116	—	—
2	1255	1320	1204	96
3	628	695	579	92
4	251	355	239	95

Hydroxy-acids do not react under these conditions, and ketonic acids react only slowly. As the addition of citrate to the dilute tissue extracts used does not give rise to the formation of ketonic acids under the conditions of our experiments, the method is sufficiently specific for our purpose.

Metabolic quotients

In most experiments the rates of reaction are expressed by the quotient

$$\frac{\text{micromol. of substrate metabolized}}{\text{mg. tissue} \times \text{hours}}.$$

In some experiments it seemed desirable to compare the rates of citric acid breakdown or formation with the rate of O_2 uptake, since citric acid plays a role in the oxidative metabolism. The quantities of citric acid metabolized are therefore expressed in some tables in μl., 1 m.-mol. citric acid being considered equivalent to 22,400μl. The rate of the metabolic reaction is expressed by the quotient

$$\frac{\mu\text{l. substrate metabolized}}{\text{mg. dry tissue} \times \text{hours}}.$$

The reaction cis*aconitic acid → citric acid*

Extraction of "aconitase". The majority of experiments were performed with extracts of pigeon breast muscle. Chilled breast muscle was minced through a Latapie mincer and ground with 5 vol. 0·1 *M* phosphate buffer of *p*H 7·4 (except where otherwise stated). Quartz (Merck's quartz sand "washed and calcined") was used to break up the tissue. The resulting suspension was rapidly centrifuged and the supernatant liquid used. This will be referred to as the "stock enzyme solution".

Aconitase is almost completely extracted by this method; a second or third extraction shows only traces of activity, as illustrated by the following experiment.

Table III. *Extraction of aconitase from muscle tissue*

1 ml. stock enzyme, 2 ml. phosphate buffer *p*H 7·4, 1 ml. *cis*aconitate (0·0081 *M*). 40°.

	Citric acid formed after 30 min. incubation μmol.
First extract	6·8
Second extract	0·9
Third extract	0·27
Fourth extract	0

The activity of the enzyme does not deteriorate within a fortnight if kept in the refrigerator.

The course of the citric acid formation from cis*aconitic acid.* To follow the rate of formation of citric acid from *cis*aconitic acid it was convenient to use dilute extracts, since the reaction is very rapid in the stock solution. Stock enzyme solution from pigeon breast muscle was diluted 50 times with 0·1 *M* phosphate

buffer (*p*H 7·4). 3 ml. dilute extract were incubated with 1 ml. *cis*aconitate (0·00735 *M*) at 40° under anaerobic conditions, and the formation of citrate was measured at various intervals.

Table IV

Time of incubation (min.)	10	20	40	60	120
Citrate formed (μmol.)	2·2	4·1	5·6	6·0	6·4

The initial rate of citrate formation, expressed in the terms of a metabolic quotient, is thus: $Q_{\text{citrate}} = 120$ μl./mg./hr.

The position of equilibrium cis*aconitic acid* $\rightleftharpoons$ *citric acid in muscle extract.* In the previous experiment the final amount of citrate represents 87 % of the added *cis*aconitate. The following table shows that in other experiments in which the reaction was allowed to reach the equilibrium, the reaction generally stopped when 75–85 % of the added *cis*aconitate had been converted into citrate.

Table V. *Stock enzyme solution diluted with 0·1* M *phosphate buffer* p*H 7·4. 40°. N_2*

No.	Dilution of stock enzyme solution	Time of incubation min.	*Cis*aconitate added μmol.	Citrate formed μmol.	% conversion
1	3	130	7·9	6·75	85
2	9	60	7·0	5·2	75
3	40	60	6·35	5·35	85
4	10	120	755	583	78
5	2	120	685	535	78

The p*H optimum of aconitase in muscle extract.* The experiments were arranged in such a way as to allow the determination of the initial rates of citric acid formation from *cis*aconitic acid by extracts of different *p*H. The curve shows a sharp maximum at *p*H 7·4. It will later be seen that the (apparent) optimum in tissue slices is at 7·9.

Table VI. p*H optimum*

3 ml. enzyme (stock enzyme diluted 60 times with buffer of appropriate *p*H) + 1 ml. *cis*-aconitate (0·01 *M*). 20 min. at 40°

*p*H	2·6	3·9	4·9	6·2	6·8	7·4	8·6	9·2
Citrate formed (μmol.)	0·27	0·2	0·7	1·2	4·1	5·0	2·8	1·5

Activity of other tissues. Intact cells. Aconitase occurs in all the animal tissues which we examined, with the exception of red blood cells. The rate of formation of citric acid from *cis*aconitic acid in various tissues is given in the following table:

Table VII. *Citric acid formation from* cis*aconitic acid by rat tissues*

Bicarbonate saline. 5 % CO_2 in N_2. 40°. Initial concentration of *cis*-aconitate = 0·025 *M*.

Tissue	Time of shaking min.	Q_{citrate} μl./mg./hr.
Liver	30	8·0
(sliced)	60	8·5
Kidney cortex	30	12·5
(sliced)	60	14·0
Testis	30	4·2
(teased)	60	5·0
Brain cortex (sliced)	60	4·6

The rate increases on raising the initial concentration of *cis*aconitic acid (Table VIII).

Table VIII. *Citric acid formation from varying concentrations of* cis*aconitic acid by rat kidney cortex slices*

Bicarbonate saline. 60 min. at 40°. 5% CO_2 in N_2.

Initial concentration *cis*aconitate	0·0033 *M*	0·014 *M*	0·025 *M*
$Q_{citrate}$	5·9	16·9	21·7

Tissue extracts. Extracts of rat tissues were made in the same way as described for pigeon breast muscle, except that the preliminary mincing was omitted. In extracts the rate of reaction was always much higher than in slices.

Table IX. *Citric acid formation from* cis*aconitic acid by rat tissue extracts*

Phosphate buffer *p*H 7·4. N_2. 60 min. at 40°. Initial concentration *cis*aconitate = 0·025 *M*.

Tissue extracted	$Q_{citrate}$ μl./mg./hr.
Kidney cortex	80·0
Liver	61·5
Lung	14·5
Submaxillary glands	11·9
Whole brain	10·0
Intestine	7·8
Testis	7·6
Red blood cells	0
Pigeon breast muscle	120·0

The slower rate of reaction observed in slices may be due to the fact that the *p*H in the tissue is not optimal for the reaction. It has been shown that aconitase in pigeon breast muscle extract shows a sharp *p*H maximum at 7·4. The following experiment shows that the *p*H optimum is different in slices (about *p*H 8·0).

Table X. *Formation of citric acid from* cis*aconitic acid by rat liver slices in bicarbonate medium of varying* p*H*

Bicarbonate saline. CO_2 in N_2. 30 min. at 40°. Initial concentration *cis*aconitate = 0·067 *M*.

*p*H	8·3	8·0	7·8	7·65
$Q_{citrate}$	54·2	75·5	71·0	55·0

It is probable that this difference is an apparent one. The concentration of bicarbonate in the tissue is known to be lower than in the medium, whilst the concentrations of free CO_2 are of the same order in medium and tissue. The *p*H of the tissue is thus lower than that of the medium. The experiment recorded in Table X does not therefore represent the real *p*H activity curve of aconitase. The latter can only be measured in extracts.

*The reaction citric acid → cis*aconitic acid

To measure the formation of *cis*aconitic acid from citric acid, pigeon breast muscle extract was incubated anaerobically at 40° with citric acid, and the resulting solution tested for the formation of aconitic acid. A small but definite quantity of aconitic acid, amounting finally to 4–5% of the added citric acid, was found; this value did not further increase on prolonged incubation.

Table XI. *Course of aconitic acid formation from citric acid*

Stock enzyme solution (diluted 12 times) in phosphate buffer *p*H 7·4. 0·02 *M* citrate. 40°.

Time of shaking (min.)	5	10	20	40
Aconitate formed (μmol.)	2·8	4·0	4·8	5·9

Citric acid added = 146 μmol.

The effect of the citric acid concentration on the formation of aconitic acid is shown in the following table:

Table XII. *Formation of aconitic acid from citric acid (final values; effect of citrate concentration)*

Pigeon breast muscle extract. *p*H 7·4. 40°.

Citrate added μmol.	Aconitate formed μmol.	% conversion
145	5·9	4·1
5020	240·0	4·8
2480	127·0	5·1
3380	150·0	4·4
4055	193·0	4·75
1000	42·0	4·2

Formation of isocitric *acid*

The following tables give the results of experiments in which the sum of the citric acid and aconitic acid remaining after incubation was compared with the amount of substrate added.

Table XIII. *Citric acid formed and aconitic acid remaining after incubation of pigeon breast muscle extract with* cisaconitic *acid*

Aconitate added μmol.	Time of incubation min.	Citrate formed μmol.	Aconitate left μmol.	Loss	% loss
754	5	87	486	181	24
754	30	450	104	100	13
754	120	582	65	107	14
685	120	536	60	89	13
1020	90	615	285	140	14

Table XIV. *Aconitic acid formed and citric acid left, after incubation of pigeon breast muscle extract with citric acid*

Citrate added μmol.	Time of incubation min.	Aconitate formed μmol.	Citrate left μmol.	Loss	% loss
2480	30	108	1680	692	28
2480	120	127	1790	563	22
3380	330	150	2780	450	13
4050	1320	193	3150	707	17
1000	90	46	830	124	12

In all experiments, the measured sum of citric and aconitic acid equivalents is less than the amount of substrate added. This indicates the formation of a third substance. From schemes put forward previously [Martius, 1937] the missing fraction was expected to be *iso*citric acid, and experiments were performed in which the development of optical rotation in the extracts after incubation with either citric or *cis*aconitic acids was measured. These experiments will not be reported in detail since they do not go beyond Martius's results [1938].

In accordance with Martius the rotation observed corresponded to 10–20% *iso*citric acid, in agreement with the view that the above mentioned deficit is due to the formation of *iso*citric acid. Our experiments also confirm Martius's finding of a rapid formation of *iso*citric acid from *cis*aconitic acid, followed by a partial disappearance (see curve 2 of Martius's paper).

Aconitase in cucumber seeds

Aconitase also occurs in cucumber seeds [Martius, 1938] and it seemed of interest to compare the enzymes from different sources. An extract of dried cucumber seeds was prepared according to the directions of Scherstén [1936]. One part of ground seed kernel was extracted with two parts 0·87% K_2HPO_4 and the centrifuged extract used.

(*a*) *Incubation with* cis*aconitic acid.* The amount of citric acid formed by incubation of this extract with *cis*aconitic acid, together with the amount of aconitic acid remaining in the solution, was measured. Table XV summarizes the results of three experiments.

Table XV. *Cucumber seed extract incubated with* cis*aconitate 40°. Octyl alcohol added*

Initial concentration of aconitic acid = 0·03 *M* approx.

No.	Vol. of enzyme ml.	Aconitate added μmol.	Citrate formed μmol.	Aconitate left μmol.	Loss	Time of incubation min.
1	15	500	237 (47%)	74·5	191 (38%)	120
2	15	607	58 (9%)	290	259 (42%)	20
		607	121 (20%)	250	235 (39%)	40
		607	168 (27%)	220	218 (36%)	60
		607	231 (27%)	177	198 (32%)	120
		607	295 (48%)	99	214 (35%)	240
3	15	540	87 (16%)	300	155 (29%)	30

(*b*) *Incubation with citric acid.* In another series of experiments cucumber seed extract was incubated with citric acid, and the amount of aconitic acid formed, together with the amount of citric acid remaining, was determined (Table XVI).

Table XVI. *Cucumber seed extract incubated with citrate at 40°. Octyl alcohol added*

Initial concentration of citrate = *M*/30. Amounts of metabolites given in μmol.

No.	Vol. of enzyme ml.	Citrate added	Aconitate formed	Citrate left	Loss	Time of incubation min.
1	15	500	21 (4·2%)	466	13 (2·6%)	30
2	15	500	23 (4·6%)	428	49 (5·8%)	60
3	15	500	22 (4·4%)	500	—	120
4	15	500	29 (5·8%)	476	—	240
5	15	500	27 (5·4%)	466	9 (1·8%)	360

From these figures it is seen that the activity of the enzyme in cucumber seed is very much lower than in muscle (compare Table IV and Tables XV and XVI). The initial velocity of the reaction in cucumber seed extract is about 1·6% of that in muscle extract. Both reactions, *cis*aconitic acid → citric acid and citric acid → *cis*aconitic acid take place in cucumber seed extract, but no true equilibrium was reached in our experiments.

There is a small deficit when citric acid is the substrate (2–6%) but a very considerable loss when *cis*aconitic acid is the substrate (32–42%). The fact that no equilibrium is established may be due to the comparatively low activity of the enzyme.

Discussion

Aconitase is an unusual enzymic system in that it forms two different isomeric compounds through addition of water to a double bond. No analogy is known to occur amongst enzymes, but many similar reactions are known to the organic chemist. For example, the addition of iodine monochloride to propylene gives $CH_3CHCl.CH_2I$ (69%) and $CH_3CHI.CH_2Cl$ (31%) [Ingold & Smith, 1931]. These reactions, however, are not reversible.

Summary

1. A method has been worked out for the quantitative determination of aconitic acid based on the catalytic hydrogenation of the double bond.

2. The action of aconitase (the conversion of citric acid into *cis*aconitic acid and the reverse reaction) has been studied in animal tissues. Aconitase is extracted with $M/10$ phosphate buffer pH 7·4 from minced pigeon breast muscle and other tissues.

3. In tissue extracts aconitase converts about 75–85% of added *cis*aconitic acid into citric acid.

4. Citric acid incubated with pigeon breast muscle extract forms about 4% aconitic acid.

5. The equilibrium is reached when the solution contains about 16% *iso*-citric acid (Martius), about 4% *cis*aconitic acid and about 80% citric acid.

6. The pH optimum of aconitase in tissue extracts is at 7·4. In tissue slices the (apparent) optimum is 7·9.

7. Aconitase was found in pigeon breast muscle, rat liver, kidney cortex, testis, brain cortex, lung, submaxillary gland, intestine, but not in red blood cells of the rat.

8. Aconitase also occurs in cucumber seeds but its activity is only 1·6% of that of pigeon breast muscle and no true equilibrium between *cis*aconitic, *iso*-citric and citric acids was reached with cucumber seed extract.

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